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|--|--|---|--|
| (51) International Patent Classification <sup>4</sup> :<br><b>C12N 15/00, A61K 39/04<br/>G01N 33/569</b>   |  | A2  | (11) International Publication Number: <b>WO 88/05823</b><br>(43) International Publication Date: <b>11 August 1988 (11.08.88)</b> |
| (21) International Application Number: <b>PCT/US88/00281</b>   |  | (74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).   |  |
| (22) International Filing Date: <b>1 February 1988 (01.02.88)</b>  |  | (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). |  |
| (31) Priority Application Number: <b>010,007</b>   |  | Published<br><i>Without international search report and to be republished upon receipt of that report.</i>  |  |
| (32) Priority Date: <b>2 February 1987 (02.02.87)</b>  |  |   |  |
| (33) Priority Country: <b>US</b>   |  |   |  |
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| (54) Title: MYCOBACTERIUM TUBERCULOSIS GENES ENCODING PROTEIN ANTIGENS   |  |   |  |

## 5.7. Abstract

*Mycobacterium tuberculosis* genes encoding five immunologically relevant proteins have been isolated by systematically screening a lambda gt11 recombinant DNA expression library with a collection of murine monoclonal antibodies directed against protein antigens of this pathogen. One of the *M. tuberculosis* antigens, a 65kD protein, has been shown to have determinants common to *M. tuberculosis* and *M. leprae*. In addition, genes encoding proteins of other mycobacteria (*M. africanum*, *M. smegmatis*, *M. bovis* BCG and *M. avium*) have been isolated. Isolation and characterization of genes encoding major protein antigens of *M. tuberculosis* make it possible to develop reagents useful in the diagnosis, prevention and treatment of tuberculosis. They can be used, for example, in the development of skin tests, serodiagnostic tests and vaccines specific for tuberculosis.

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MYCOBACTERIUM TUBERCULOSIS GENES AND  
ENCODING PROTEIN ANTIGENS

Description

Background

05      Tuberculosis was the major cause of infectious mortality in Europe and the United States in the 19th and early 20th centuries. Dubos, R. and J. Dubos, The White Plague: Tuberculosis, Man and Society, Little Brown & Co., Boston, MA, (1952).  
10      Today, it remains a significant global health problem.

15      For example, in the United States there are over 20,000 new cases of tuberculosis diagnosed annually. In addition, the steadily declining incidence of tuberculosis evident in preceding years appears to have changed course, reaching a plateau in 1985 and showing an increase in the first half of 1986. Centers for Disease Control, Morbidity/Mortality, Weekly Report, 34:774 (1986); and Centers for Disease Control, Morbidity/Mortality, Weekly Report, 35:774 (1986).

20      Worldwide, tuberculosis remains widespread and constitutes a health problem of major proportions, particularly in developing countries. The World Health Organization estimates that there are ten million new cases of active tuberculosis per year and an annual mortality of approximately three

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million. Joint International Union Against Tuberculosis and World Health Organization Study Group, Tubercle, 63:157-169 (1982).

05      Tuberculosis is caused by Mycobacterium (M.) tuberculosis or Mycobacterium (M.) bovis, which are the 'tubercle bacilli' of the family Mycobacteriaceae. M. bovis is a species which causes tuberculosis in cattle and is transmissible to humans and other animals, in whom it causes tuberculosis. At 10 present, nearly all tuberculosis is caused by respiratory infection with M. tuberculosis. Infection may be asymptomatic in some, but in other individuals, it produces pulmonary lesions which lead to severe debilitation or death. Resistance to 15 tuberculosis is provided by cell-mediated immune mechanisms.

20      Mycobacteria are aerobic, acid-fast, non-spore-forming, non-motile bacilli with high lipid contents and slow generation times. M. leprae is the etiologic agent of leprosy and, among the other mycobacteria, the only major pathogen. Bloom, B.R. and T. Godal, Review of Infectious Diseases, 5:765-780 (1983). However, other mycobacterial species are capable of causing disease. Wallace, R.J. et.al., 25 Review of Infectious Diseases, 5:657-679 (1984). M. avium, for example, causes tuberculosis in fowl and in other birds. Members of the M. Avium-intracellularare complex have become important pathogens among individuals with acquired immunodeficiency syndrome (AIDS). Certain groups of 30

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individuals with AIDS have a markedly increased incidence of tuberculosis as well. Pitchenik, A.E. et. al., Annals of Internal Medicine, 101:641-645 (1984).

05 Diagnostic and immunoprophylactic measures for mycobacterial diseases have changed little in the past half century. Tuberculin, developed by Koch as a cure for tuberculosis in the late 1800s, is an M. tuberculosis filtrate of complex and poorly-defined composition. It is used as a skin test antigen to detect prior exposure to the bacillus. Enrichment of the protein fraction of this material in the 1930's produced the purified protein derivative (PPD) which is still used to diagnose exposure to tuberculosis. Seibert, F.M. et.al., American Review of Tuberculosis, 30(Suppl.):705-778 (1934). Its usefulness is limited, however, by its lack of specificity and its inability to distinguish active disease from prior sensitization by contact with M. tuberculosis or cross-sensitization to other mycobacteria. Young, R.A. and R.W. Davis, Proceedings of the National Academy of Sciences, USA, 80:194-1198 (1983).

20 Bacille Calmette Guerin (BCG), an avirulent strain of M. bovis, has been used widely as a live vaccine against tuberculosis for over 50 years. Calmette, A., C. et.al., Bulletin of the Academy of

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05                   Medicine Paris, 91:787-796 (1924). During that time, numerous studies have shown that BCG has protective efficacy against tuberculosis. These studies are reviewed by F. Luelmo in American Review of Respiratory Diseases, 125(pt. 2):70-72 (1982). However, more recently, a major trial of BCG in India indicated that such a vaccine was not protective against tuberculosis in this setting. World Health Organization WHO Technical Report Series, 651 (1980). Presently available approaches to diagnosing, preventing and treating tuberculosis are limited in their effectiveness and must be improved if a solution is to be found for the important public health problem tuberculosis represents

10                   worldwide.

15

#### Summary of the Invention

20                   The present invention is based on the isolation of genes encoding immunogenic protein antigens of the tubercle bacillus Mycobacterium tuberculosis (M. tuberculosis). Genes encoding such protein antigens have been isolated from a recombinant DNA expression library of M. tuberculosis DNA. Genes encoding proteins of four additional mycobacteria have also been isolated and restriction maps produced.

25

In particular, genes encoding five immunodominant protein antigens of the tuberculosis bacillus (i.e., those M. tuberculosis proteins of molecular weight 12,000 daltons (12kD), 14kD, 19kD, 65kD and 71kD have been isolated by probing a lambda gt11 expression library of M. tuberculosis DNA with

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monoclonal antibodies directed against M.  
tuberculosis-specific antigens.

Recombinant DNA clones producing the specific  
antigenic determinants recognized by the monoclonal  
05 antigens were also isolated in this manner. DNA  
from such recombinant lambda gt11 clones was mapped  
with restriction endonucleases; the restriction maps  
for genes encoding the five immunodominant protein  
antigens (i.e., genes encoding the 12kD, 14kD, 19kD,  
10 65kD and 71kD proteins) were deduced. The nucleo-  
tide sequence of three of the genes have been deter-  
mined and, in each case, the amino acid sequence of  
the encoded protein has been deduced.

Brief Description of the Drawings

Figure 1 shows restriction maps of M. tuberculosis DNA. Recombinant DNA clones isolated with  
monoclonal antibodies directed against the 12kD,  
14kD, 19kD, 65kD and 71kD protein antigens were  
mapped with restriction endonucleases. The insert  
20 DNA endpoints are designated left (L) or right (R)  
in relation to lac Z transcripts which traverse the  
insert from right to left. Restriction sites are  
represented as follows: A, Sal I; B, BamHI; E,  
EcoRI; G, BglIII; K, KpnI; P, PvuI; S, SacI; X, XhoI.  
25

Figure 2 shows arrays of antigens from M.  
tuberculosis recombinant DNA clones probed with  
rabbit hyperimmune serum. The code of the recombi-  
nant DNA clones shown on the numbers filter is: 1,  
Y3275; 2, Y3274; 3, Y3279; 4, Y3277; 5, Y3247; 6,  
30 Y3272; 7, Y3150; 8, Y3254; 9, Y3147; 10, Y3163; 11,

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Y3179; 12, Y3191; 13, Y3252; 14, Y3178; 15, Y3180; 16, Y3143; 17, lambda gt11. Clones 1, 5, 6, 7, 9 and 16 are M. tuberculosis recombinants described in the following section. Clones 10, 11, 14 and 15 are 05 M. leprae recombinants expressing epitopes of the 18kD, 28kD, 36kD and 65kD antigens, respectively. Clones 2, 3, 4, 8, 12, 13 are uncharacterized recombinants from the lambda gt11 M. tuberculosis and M. leprae libraries. Clone 17 is a non-recombinant lambda gt11 control.

Figure 3 shows arrays of recombinant mycobacterial antigens probed with monoclonal antibodies to assess the extent of cross-reactivity between recombinant protein antigen of M. tuberculosis and 15 of M. leprae. The array of clones is identical to that shown in Figure 2. Antibody probes and the antigen sizes recognized are: 1, IT-11 (71kD); 2, IT-31 (65kD); 3, IT-16 (19kD); 4, IT-1 (14kD); 5, IT-3 (12kD).

20 Figure 4 shows restriction maps of DNA encoding four proteins (71kD, 65kD, 19kD and 14kD) of M. tuberculosis and four proteins (71kD, 65kD, 19kD and 14kD) of M. bovis BCG. Restriction sites are represented as follows: A, AatII; B, BamH1; C, BcII; D, DraIII; E=EcoRI; G, BglIII; H, HinFI; K, KpnI; P, PstI; S, SalI; V, PvuI and X, XhoI.

25 Figure 5 is a comparison of restriction maps of the gene encoding the 65kD protein of 6 mycobacteria (M. leprae, M. tuberculosis, M. africanum, M. bovis BCG, M. smegmatis, M. avium). Restriction sites are 30

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as follows: B, BamH1; K, KpnI; N, SacI; P, PvuI; S, SalI; X, XhoI.

Figure 6 is the nucleotide sequence of the region containing the M. tuberculosis 19kD gene. 05 The deduced amino acid sequence of the encoded protein is also represented (protein start position, nucleotide 1110; protein stop position, nucleotide 1586).

Figure 7 is the nucleotide sequence of the 10 region containing the M. tuberculosis 71kD gene and the deduced amino acid sequence of the encoded protein.

Figure 8 is the nucleotide sequence of the 15 region containing the M. tuberculosis 65kD gene. The deduced amino acid sequences of the two long open reading frames are presented in one letter code over (540) or under (517) the appropriate triplets.

#### Detailed Description of the Invention

The invention described herein is based on the 20 isolation of genes encoding immunogenic protein antigens of the bacillus M. tuberculosis, which is the major etiologic agent of tuberculosis. In particular, it is based on the isolation, using monoclonal antibodies directed against M. tuberculosis-specific antigens, of genes encoding 25 five immunogenic protein antigens of the tuberculosis bacillus; these five antigens are immunodominant. Immunogenic antigens are those which elicit a response from the immune system. 30 Immunodominant protein antigens are immunogenic

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antigens against which the immune system directs a significant portion of its response. Genes encoding M. tuberculosis antigens of molecular weight 12,000 daltons (12kD), 14kD, 19kD, 65kD and 71kD were  
05 isolated in this manner.

Isolation and characterization of major protein antigens of M. tuberculosis, as described herein, make it possible to develop more effective tools for the prevention, diagnosis, and treatment of tuberculosis.  
10 Identification and isolation of genes encoding five immunodominant M. tuberculosis protein antigens, as well as of the five protein antigens, are described below; uses of the genes and encoded products are also described.  
15

M. bovis BCG DNA clones were also isolated for the genes encoding the 71kD, 65kD, 19kD and 14kD proteins. In order to compare M. bovis BCG and M. tuberculosis genes encoding proteins of similar molecular weight, restriction endonuclease maps were  
20 determined for DNA segments containing each of the genes. Restriction maps for each of these genes is represented in Figure 4.

In addition, DNA clones were isolated for the genes encoding the 65kD protein from M. africanum,  
25 M. smegmatis and M. avium. Restriction endonuclease maps were determined for DNA segments containing each of these genes. The restriction maps for these genes, as well as for the genes encoding the 65kD protein of M. tuberculosis, M. bovis BCG and M. leprae, are represented in Figure 5.  
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I. Construction of a recombinant expression library of M. tuberculosis DNA

A recombinant DNA expression library of M. tuberculosis DNA was constructed using lambda gt11.

05 The library was constructed with M. tuberculosis genomic DNA fragments in such a way that all protein-coding sequences would be represented and expressed. Young, R.A., B.R. Bloom, C.M. Grosskinsky, J. Ivanyi, D. Thomas and R.W. Davis, 10 Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985).

Lambda gt11 is a bacteriophage vector which is capable of driving the expression of foreign insert DNA with E. coli transcription and translation signals. Lambda gt11 expresses the insert DNA as a fusion protein connected to the E. coli Beta-galactosidase polypeptide. This approach ensures that the foreign DNA sequence will be efficiently transcribed and translated in E. coli. This approach is also useful in addressing the problem of the highly unstable nature of most foreign proteins; fusion proteins are often more resistant to proteolytic degradation than is the foreign polypeptide alone. Lambda gt11 and the E. coli strain used (Y1090) have been described previously. Young, R.A. et al., Proceedings of the National Academy of Sciences, USA, 80:1194-1198 (1983); Young, R.A. and R.W. Davis, Science, 222:778-782 (1983). The teachings of these publications are incorporated herein by reference. The library constructed in this manner has a titer of  $1 \times 10^{10}$  pfu/ml. and

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contains approximately 40% recombinants with an average insert size of 4kB.

II. Screening of the lambda gt11 M. tuberculosis library with antibody probes

05 Murine monoclonal antibodies to protein antigens of M. tuberculosis were used individually to probe the M. tuberculosis recombinant DNA library. This work is described below and with specific reference to the 65kD antigen in the Exemplification. The antibodies used as probes and the sizes of the antigens to which they bind are shown below.

|    |                 | <u>M. tuberculosis</u> |
|----|-----------------|------------------------|
|    | <u>Antibody</u> | <u>Antigen</u>         |
|    | IT-3            | 12kD                   |
| 15 | IT-20           | 14kD                   |
|    | IT-19           | 19kD                   |
|    | IT-27           | 19kD                   |
|    | IT-17           | 23kD                   |
|    | IT-29           | 23kD                   |
| 20 | IT-15           | 38kD                   |
|    | IT-21           | 38kD                   |
|    | IT-23           | 38kD                   |
|    | IT-13           | 65kD                   |
|    | IT-31           | 65kD                   |
| 25 | IT-33           | 65kD                   |
|    | IT-11           | 71kD                   |

Engers, H.D. et al., Infectious Immunology, 51:718-720 (1986).

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All monoclonal antibodies were used at approximately 1:200 to 1:300 dilution in 50mM Tris-HCl pH8/150 mM NaCl/.05% Tween 20.

05 Screening of the lambda gt11 recombinant DNA library was performed as described by Young *et al.* in Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985), the teachings of which are incorporated herein by reference. One modification was made in the method described by Young and  
10 co-workers: 1% bovine serum albumin was used in place of 20% fetal calf serum to decrease background.

15 Briefly, cloned lambda gt11 recombinants were arrayed on lawns of E. coli Y1090. The phage were grown, antigen expression was induced and the antigens were blotted and probed with serum. Detection of signal-producing plaques was performed with a biotinylated secondary antibody system (Vectastain, Vector Laboratories, Burlingame, CA) or  
20 with an alkaline phosphatase conjugated secondary antibody system (Protoblot, Promega Biotec, Madison, WI), both used according to manufacturer's instructions. Signal-producing clones were isolated using antibodies directed against protein antigens of  
25 molecular weight 12kD, 14kD, 19kD and 65kD and 71kD. In each case, similar numbers of clones were isolated in screens of approximately  $10^5$  recombinant plaques. DNA clones encoding the 23kD and 38kD antigens could not be detected with these antibodies, possibly because the native epitope is modified or topographically complex, or because the  
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antigen-antibody interaction is too weak to be recognized by current detection methods.

III. Probing of Arrays of lambda gt11 DNA  
Clones with Antibody Probes

05        0.2 ml of a saturated culture of Y1090 was added to 2.5 ml of molten LB soft agar, poured onto 100 mm plates containing 1.5% LB agar and allowed to harden at room temperature for 10 min. 100 ul of phage plate stock containing approximately  $10^{11}$  pfu/ml of the lambda gt11 DNA clones of interest were placed into alternate wells of 96-well tissue culture plates. A multi-pronged transfer device was placed briefly in the wells containing phage and then touched lightly to the surface of the plate onto which the soft agar had been poured. The plates were then incubated at 42°C for approximately 3 hours, at which point clear plaques approximately 5mm in diameter were visible. The plates were then overlayed with nitrocellulose filters saturated with 20 10mM isopropylthiogalactoside (IPTG) and incubated at 37°C for 3.5 hours. Subsequent processing of filters for detection of antigen was identical to the procedures described for screening of lambda gt11 library with antibody probes.

25        Immunoscreening of the lambda gt11 library to isolate clones reactive with monoclonal antibodies specific for the 65kD antigen is described in the Exemplification.

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#### IV. Recombinant DNA Manipulation

DNA from recombinant lambda gt11 clones was isolated and mapped with restriction endonucleases by standard techniques. Davis, R.W. et al.,  
05 Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980).

Figure 1 shows the genomic DNA restriction map deduced for each of the genes encoding the five M. tuberculosis antigens and illustrates how each of  
10 the cloned DNAs aligns with that map. All clones isolated with monoclonal antibodies directed against any single antigen align with a single genomic DNA segment. This indicates that all clones were  
15 isolated because they express the protein of interest rather than an unrelated polypeptide containing a similar or identical epitope. In addition, this result suggests that each antigen is the product of a single gene.

The orientation of each DNA insert in the recombinant clones was determined by restriction analysis. Only among the clones for the 65kD antigen were the inserts found in both possible orientations relative to the direction of lac Z transcription in lambda gt11. This suggests that  
20 this protein can be expressed in E. coli from signals independent of those provided by lac Z. Similar results have been obtained for recombinant DNA clones encoding the 65kD antigens of M. bovis and M. leprae. Thole, J.E.R. et al., Infectious Immunology, 50:800-806 (1985); Young, R.A. et al.,  
25 Nature, 316:450-452 (1985).  
30

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The nucleotide sequences of three regions of the M. tuberculosis DNA were determined: 1) the region containing the M. tuberculosis 19kD gene; 2) the region containing the M. tuberculosis 71kD gene; and 3) the region containing the 65kD gene. The three sequences are represented in Figures 6-8. Sequences were determined using standard techniques, which are described in the Exemplification.

10        V. Filter hybridization of Insert DNA

Arrays of lambda gt11 clones were created as described above and incubated at 42° for 5 hours. The plates were then overlayed with nitrocellulose filters and placed at 4°C for 1 hour. Probe DNA was labelled with <sup>32</sup>P by nick translation. Filter 15 hybridization was performed as described by Davis et al. in Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980), the teachings of which are incorporated herein by reference. Hybridization conditions were as follows: 50% 20 v/v formamide, 5x SSPE (1x SSPE is 0.18M NaCl, 10mM Na<sub>1.5</sub>H<sub>1.5</sub>PO<sub>4</sub>, 1mM Na<sub>2</sub>EDTA, pH 7.0), 1x Denhardt's solution (0.02% w/v Ficoll, 0.02% w/v polyvinyl-pyrrrolidone, 0.02% w/v bovine serum albumin), 0.3% NaDodSO<sub>4</sub> at 42°C for approximately 16 hours, followed by washing in 2x SSPE, 0.2% NaDodSO<sub>4</sub> at 45°C.

25        VI. Recombinant Antigens Recognized by Rabbit Serum

The response of a second animal to an antigen preparation of M. tuberculosis was assessed by

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examining the reactivity of rabbit anti-M. tuberculosis hyperimmune sera with recombinant antigens. Cloned lambda gt11 recombinants were arrayed on lawns of E. coli and probed with the rabbit hyperimmune serum. Anti-M. tuberculosis hyperimmune serum, produced by repeated immunization of rabbits with M. tuberculosis H37Rv culture filtrate, was provided by J. Bennedsen (Statens Serum Institut, Copenhagen, Denmark). These sera were used at 1:100 dilution.

These sera produced positive signals with lambda gt11 clones encoding each of the five M. tuberculosis epitopes which had been isolated with murine monoclonal antibodies (Figure 2). Particularly strong signals were observed with the 65kD and 71kD antigens (Figure 2). These results demonstrate that mice and rabbits can mount an antibody response to the same protein antigens of M. tuberculosis.

Clones for the five M. tuberculosis antigens were detected at similar frequencies in the lambda gt11 recombinant DNA library. Thus, the number and type of antigen-producing clones isolated with polyclonal serum antibodies should reflect the relative titer and diversity of the individual antibodies in this serum.

To determine whether any of the 5 M. tuberculosis antigens are relatively immunodominant in the rabbit humoral immune response to M. tuberculosis, the M. tuberculosis lambda gt11 recombinant DNA library was screened with the rabbit serum. Forty signal-producing clones were isolated, arrayed on

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lawns of E. coli Y1090 and probed with monoclonal antibodies directed against each of the 5 recombinant M. tuberculosis protein antigens. Remarkably, 17 of the 40 clones (43%) reacted strongly with at least one of the four anti-65kD monoclonal antibodies tested. An additional six clones (15%) reacted strongly with the anti-17kD monoclonal antibody (IT-11). This indicates that a large proportion of the anti-M. tuberculosis antibody present in the rabbit serum was directed against the 65kD antigen of M. tuberculosis and suggests that it is a dominant antigen for the rabbit humoral immune response. Seventeen of the clones did not react with any of the monoclonal antibodies tested, suggesting that the rabbit sera may identify M. tuberculosis proteins not recognized by the murine antibodies.

VII. Antigenic Relatedness of M. tuberculosis and M. leprae Proteins

There is evidence that M. tuberculosis and M. leprae share immunologically important antigens. To assess this further, an investigation of the exact nature of the immunological relatedness among recombinant protein antigens of M. tuberculosis and M. leprae was conducted.

For each of five M. tuberculosis and four M. leprae protein antigens, a single recombinant DNA clone containing most or all of the gene of interest was used to express antigen in the following manner. The recombinant phage clones were arrayed on a lawn

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of E. coli Y1090, which was then grown and induced for antigen expression.

Antigen immobilized on nitrocellulose filters was then probed with 26 individual anti-M. tuberculosis and M. leprae monoclonal antibodies. Figure 3 shows the array of DNA clones used and the results obtained with the anti-M. tuberculosis antibodies IT-1, IT-3, IT-11, IT-16, and IT-31, which recognized proteins of 14kD, 12kD, 71kD, 19kD and 65kD respectively. Table 1 details the full results of these cross-screening experiments, showing the reactivity of antigen expressed from individual recombinant DNA clones with each of the individual monoclonal antibodies. Clones were scored as positive only if the signal produced was clearly greater than the background signal produced by the non-recombinant lambda gt11 clone included in each array.

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**TABLE 1**  
**Reactivity of Monoclonal Antibodies with**  
**Recombinant Protein Antigens**

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Several conclusions can be drawn from the results shown in Table 1. Among the 11 monoclonal antibodies that recognize a 65kD antigen, 7 react with the 65kD protein from both mycobacteria (IT-31, 05 C1.1, IIH9 (identical to IT-33), IIC8, T2.3, Y1-2, SA2.D7C), one antibody reacts only with the M. tuberculosis 65kD protein (IT-13), and two antibodies react only with the M. leprae 65kD protein (IIIE9 and IIIC8). One antibody, ML30A, 10 cross-reacts with an antigen in E. coli and could not specifically identify antigen-producing clones. These results indicate that the 65kD protein antigens of M. tuberculosis and M. leprae are homologues and share a number of epitopes. In 15 addition to these shared epitopes, however, both 65kD antigens have epitopes that are specific for one species relative to the other.

No cross-reactivity was observed between other antigens of these two mycobacterial species. 20 Because monoclonal antibodies recognize a single epitope and because only one or a few antibodies were available for each antigen, it is not clear whether the 65kD proteins are the only homologous protein antigens of M. tuberculosis and M. leprae. 25 Among the antigens for which lambda gt11 clones have been isolated, the 18kD antigen of M. leprae and the 19kD antigen of M. tuberculosis are of similar size. To determine whether these two antigens are related, 30 the homology of the DNA sequences that encode these antigens was examined. At conditions of moderate stringency, no hybridization was observed between

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the insert DNA and Y3147 (an M. tuberculosis 19kD clone) and Y3179 (an M. leprae 18kD clone). This indicates no significant homology between the DNA sequences of the insert DNAs of these two clones.  
05 This result suggests that the M. tuberculosis 19kD and the M. leprae 18kD proteins are unlikely to be homologous.

As a result of the work described, recombinant DNA clones encoding five major protein antigens of M. tuberculosis were isolated through the use of an extensive collection of well-characterized murine monoclonal antibodies. These five proteins were also found to be major antigens in the rabbit humoral immune response to M. tuberculosis. One of 10 these antigens, the 65kD protein, is shared with another other mycobacterial pathogen M. leprae.  
15

Several lines of evidence indicate that the 65kD antigen is among the most immunodominant of the protein antigens of M. tuberculosis. Eleven of the 20 25 different M. tuberculosis and M. leprae monoclonal antibodies examined in this study recognized the 65kD recombinant antigen from one or both mycobacteria. In addition, almost half of the recombinant DNA clones isolated with rabbit poly- 25 30 clonal anti-M. tuberculosis sera express the 65kD antigen, reflecting the predominance of antibody to this antigen in these sera.

Considerable evidence indicates that the 65kD antigen plays an important role in the human response to tuberculosis. Antibodies directed against 30 this protein can be detected in the serum of

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patients with tuberculosis. The 65kD antigen is present in purified protein derivatives (PPD's) of M. tuberculosis, M. bovis, and other mycobacteria. Thole, J.E.R. et al., Infection Immunity, 50:800-806 05 (1985). Finally, helper T cell clones reactive with recombinant 65kD antigen have been isolated from patients with tuberculosis, indicating that this antigen is involved in the cell-mediated as well as the humoral immune response to tuberculosis.

Among the major antigens of the leprosy bacillus, the 65kD antigen appears to elicit antibody and T cell responses similar to those observed for the M. tuberculosis antigen. Both serum antibodies and T cells directed against the 65kD M. leprae antigen have been observed in patients with leprosy. Britton, W.J. et al., Journal of Immunology, 135:4171-4177 (1985); Mustafa, A.S. et al., Nature, 319:63-66 (1986). In addition, T cell clones from leprosy patients have been found to respond to recombinant 65kD protein of M. bovis, as well as to PPD's from both M. bovis BCG and M. leprae. Emmrich, F. et al., Journal of Experimental Medicine, 163:1024-1029 (1986); Shankar, P. et al., Journal of Immunology, 136:4255-4263 (1986). It is interesting to note that in vaccine trials in Asia and Africa, BCG provided significant protection against leprosy, ranging from 20% to 80%. Fine, P., Tubercle, 65:137-153 (1984). An intriguing possibility is that the M. bovis BCG 65kD antigen is involved in engendering the immune protection 10 15 20 25 30

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provided by this vaccine against M. leprae, as well as against M. tuberculosis.

05 In addition to the 65kD antigen, there is evidence that the 19kD and 71kD antigens of M. tuberculosis may be particularly important in the immune response to this bacillus. Helper T cell clones from tuberculosis patients have been isolated which respond to the recombinant 19kD protein. The 71kD antigen is recognized by the humoral immune system of both mice and rabbits, and antibody to this antigen has been shown to be a prominent component of hyperimmune anti-M. tuberculosis rabbit sera.

15 VIII. Isolation of DNA Clones for Genes Encoding Proteins of Additional Mycobacteria

Using the procedures described above for isolation of genes encoding M. tuberculosis proteins, genes encoding proteins of additional mycobacteria were isolated. DNA clones containing 20 genes encoding the following proteins were isolated:

| <u>Mycobacterium</u> | <u>Protein</u> | <u>Clone</u> |
|----------------------|----------------|--------------|
| <u>M. bovis</u> BCG  | 71kD           | PL1-101      |
|                      | 65kD           | PL1-105      |
|                      | 19kD           | PL1-501      |
|                      | 14kD           | PL1-502      |
| <u>M. smegmatis</u>  | 65kD           | PL1-206      |
| <u>M. avium</u>      | 65kD           | PL1-401      |
| <u>M. africanum</u>  | 65kD           | PL1-301      |

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For purposes of comparison, genes encoding the following proteins were isolated for M. tuberculosis and M. leprae:

|    | <u>Mycobacterium</u>   | <u>Protein</u> | <u>Clone</u> |
|----|------------------------|----------------|--------------|
| 05 | <u>M. tuberculosis</u> | 71kD           | Y3272        |
|    |                        | 65kD           | Y3150        |
|    |                        | 19kD           | Y3147        |
|    |                        | 14kD           | Y3248        |
|    | <u>M. leprae</u>       | 65kD           |              |

10 The following strains were used for this purpose:

|    | <u>Species</u>         | <u>Isolate</u>           |
|----|------------------------|--------------------------|
|    | <u>M. leprae</u>       | Armadillo isolate (WHO)  |
| 15 | <u>M. tuberculosis</u> | Erdmann strain           |
|    | <u>M. africanum</u>    | African clinical isolate |
|    | <u>M. bovis</u> BCG    | Danish vaccine strain    |
|    | <u>M. smegmatis</u>    | MC <sup>2</sup> -6       |
|    | <u>M. avium</u>        | AIDS patient isolate     |

20 DNA from recombinant lambda gt11 clones was isolated, as described above, and mapped with restriction endonucleases, using standard techniques. Davis, R.W. et al., Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980).

25 Figure 4 presents a comparison of the restriction maps for four genes of M. tuberculosis with the restriction maps for four genes of M. bovis BCG which encode proteins of the same molecular weight. As is evident from the figure, in each case, the

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restriction sites on the two genes (e.g., those on the M. tuberculosis gene and those on the M. bovis gene which encodes a protein of the same molecular weight) are essentially identical. This indicates  
05 that the sequence of the genes of the two mycobacteria (at least those encoding these four proteins) are very similar and, therefore, the proteins they encode are also very similar.

Figure 5 presents a comparison of the restriction map for the gene encoding the 65kD protein for the six mycobacteria. As is evident, the restriction maps for the genes encoding the 65kD protein of M. tuberculosis, M. africanum, M. bovis BCG, M. smegmatis and M. avium are essentially identical.  
10

15 The fact that there is no detectable difference among these mycobacteria at the level of the restriction map is an indication that, at least at this level, the encoded proteins are the same.

As is also evident, the map of the M. leprae 20 65kD gene has several identical restriction sites in common with those of the other mycobacteria; it also has two sites not found in the other genes and lacks three sites present in the others. This indicates that, at the level of the restriction map, there are similarities in the DNA (and the encoded protein).  
25 In addition, however, there are differences apparent at this level.

#### IX. Diagnostic, Therapeutic and Preventive Applications

30 The isolation of genes encoding major protein antigens of M. tuberculosis makes it possible to

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address problems which presently exist in diagnosing  
treating and preventing tuberculosis. Isolation of  
genes encoding proteins of other mycobacteria, such  
as M. bovis BCG, M. africanum, M. smegmatis and M.  
05 avium makes it possible to address similar problems  
in diseases which they cause.

The nucleotide sequence of three of the five  
genes has been determined. The sequence of the  
remaining genes can be determined using well-known  
10 methods, such as that of Sanger et al. Sanger, F.  
et.al., Proceedings of the National Academy of  
Sciences, USA, 74:5463-5467 (1977). The amino acid  
sequence of each of the immunodominant proteins has  
been deduced from the nucleotide sequence of the  
15 three genes and can be done for the others.

Identification and characterization of the  
genes for major tuberculosis protein antigens and of  
the proteins themselves make it possible to develop  
improved reagents for diagnosis and immuno-  
20 prophylaxis of tuberculosis. Proteins antigens  
encoded by an entire gene, or amino acid sequences  
(e.g., peptides, protein fragments) which make up  
the antigenic determinant of a M. tuberculosis  
antigen (i.e., M. tuberculosis-specific antigenic  
25 determinants) may be used in serodiagnostic tests  
and skin tests. Such antigens would be highly  
specific to the tuberculosis bacillus and the tests  
in which they are used would also be highly  
specific. Highly specific serological tests would  
30 be of great value in screening populations for

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individuals producing antibodies to M. tuberculosis-specific antigenic determinants; in monitoring the development of active disease in individuals and in assessing the efficacy of treatment. As a result,  
05 early diagnosis of tuberculosis will be feasible, thus making it possible to institute treatment at an early stage of the disease and, in turn, to reduce the likelihood it will be transmitted.

As a result of the work described, it is also  
10 possible to determine which segment(s) of the M. tuberculosis antigen is recognized by M. tuberculosis-specific T cells. A mixture of peptides recognized by helper T cells can serve as a specific skin test antigen useful in assessing  
15 immunological status (delayed hypersensitivity) of infected individuals and those with whom they come in contact. This specific skin test antigen would be useful in evaluating rapidly the immunological efficacy of anti-tuberculosis vaccines.  
20

It is reasonable to expect that the products encoded by M. tuberculosis genes, particularly those shown to be recognized by helper T cells, are themselves immunogenic and thus useful components of vaccines against tuberculosis. These products  
25 include proteins and portions of such proteins (e.g., polypeptides and peptides). For example, one approach to vaccine development is the introduction of genes encoding products (e.g., polypeptides) which provide immunological protection into viruses  
30 such as vaccinia virus, or bacteria, such as cultivatable mycobacteria, thus producing a vaccine

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capable of engendering long-lasting and very specific immunity. The genes encoding five immunodominant protein antigens of the tuberculosis bacillus, described herein, are useful for that  
05 purpose; genes encoding the 65kD, 19kD and 71kD antigens, or a portion thereof, are particularly valuable in vaccine construction.

Because of the similarities in the DNA encoding similarly-sized proteins and, thus, of the encoded 10 proteins themselves, it is possible that, for example, a vaccine effective against two or more of the mycobacteria can be produced.

#### EXEMPLIFICATION

##### Isolation and Analysis of Recombinants Expressing the 65kD M. tuberculosis Antigen

The recombinant DNA library of M. tuberculosis genomic DNA fragments in the lambda gt11 vector was constructed as described above. Recombinant phage lambda RY3143 and lambda RY3146 were used. Young,  
20 R.A. et al., Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985). Subclones of the mycobacterial DNA inserts in these recombinant phage were constructed in pUC19 or M13mp9 vectors using standard recombinant DNA techniques. Messing,  
25 J. and J. Viera, Gene, 19:269-276 (1982). Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

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Monoclonal antibodies specific for the 65kD antigen were obtained from the Immunology of Tuberculosis Scientific Working Group under a grant from the WHO/World Bank/UNDP Special Program for  
05 Vaccine Development. These antibodies included IT-13 (WTB-78), IT-31 (SA2D5H4), and IT-33 (MLIIH9). Coates, A.R.M. et al., Lancet, 2:167-169 (1981). Gillis, T.P. and T.M. Buchanon, Immunology, 37:172-178 (1982). Anti-B-galactosidase antibodies  
10 were purchased from CooperBiomedical. Polyclonal rabbit antisera directed against a sonicate of M. tuberculosis strain H37Rv were elicited as described by Minden and co-workers. Minden, P. et al., Infect. Immun., 46:519-525 (1984). Results are  
15 shown in Table 2.

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TABLE 2: PATTERNS OF ANTIBODY REACTIVITIES<sup>a</sup>

|    | <u>Number of Clones</u> | <u>Reactivity with Antibodies</u> |              |              |
|----|-------------------------|-----------------------------------|--------------|--------------|
|    |                         | <u>IT-13</u>                      | <u>IT-31</u> | <u>IT-33</u> |
| 05 | 27                      | +                                 | +            | +            |
|    | 1                       | +                                 | +            | +            |
|    | 2                       | +                                 | -            | +            |
|    | 3                       | -                                 | +            | +            |
|    | 1                       | +                                 | -            | -            |
|    | 2                       | -                                 | +            | -            |
| 10 | 2                       | -                                 | -            | +            |

<sup>a</sup>: Recombinant clones expressing antigens reactive with the 65kD antigen specific monoclonal antibodies IT-13, IT-31, and IT-33 were isolated as described above. For the initial screen, a pool of the three 15 antibodies was used; it contained a 1:1000 dilution of each antibody to screen a total of about  $8 \times 10^5$  recombinant phage from the lambda gt11-M. tuberculosis library. To determine which monoclonal antibody reacted with which of the 38 plaque-20 purified recombinants, about 100 pfu of each recombinant phage were inoculated in small spots on a lawn of Y1090. The phage were allowed to grow and induced to synthesize the foreign proteins as described previously. The filters were then reacted 25 with a 1:1000 dilution of one of the monoclonal hybridoma antibodies as described above.

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The lambda gt11-M. tuberculosis library was screened with the monoclonal antibodies specific for the 65kD antigen and clones reactive with them were isolated essentially as described by Young et al.

05 Young, R.A. et al., Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985). Briefly, for each 150mm LB plate, 0.6ml of a fresh overnight culture of Y1090 was infected with 1-2  $\times 10^5$  plaque forming units of the library. After  
10 3.5-4 hours of growth at  $42^\circ\text{C}$ , the plaques were overlaid with a dry nitrocellulose filter which had been saturated with 10mM isopropyl-B-D-thiogalacto-pyranoside (IPTG). The plates were incubated an additional 3.5-4 hours at  $37^\circ\text{C}$  and then removed to  
15 room temperature and the position of the filters marked. The filters were washed briefly in TBST (50 mM Tris-HCl, pH 8, 150mM NaCl, 0.05% Tween 20) and then incubated in TBST + 20% fetal calf serum.  
After 30 minutes at room temperature, the filters  
20 were transferred to TBST plus antibody. For the initial screen, the antibody mix contained a 1:1000 dilution of IT-13, IT-31, and IT-33. The filters were incubated with the antibody solution overnight at  $4^\circ\text{C}$  with gentle agitation, washed in TBST and  
25 reacted with biotinylated goat anti-mouse immuno-globulin, the Vectastain ABC reagent, and developer as described by the manufacturer (Vector Laboratories). After the color had developed the filters were washed with several changes of water  
30 and air dried. Phage corresponding to positive signals were twice plaque purified. To determine

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which monoclonal antibodies reacted with which of  
the recombinant phage, about 100 pfu of a purified  
phage stock were inoculated in a small spot on a  
lawn of Y1090 bacteria on an LB plate. The phage  
05 were allowed to grow and induced to synthesize the  
foreign proteins as described above. The filters  
were then reacted with a 1:1000 dilution of one of  
the monoclonal antibodies. The subsequent steps  
were the same as for the initial screen.

10 Western blot assays were carried out as  
follows: Cells containing phage or plasmids in  
which the expression of the foreign sequences was  
under the control of the E. coli lac gene regulatory  
sequences were induced to synthesize the foreign  
15 proteins by incubating the cells in the presence of  
2.5mM IPTG for 2 hours. Crude lysates of cells  
expressing lambda gt11 recombinants were made as  
described in Huynh et al. Huynh, T.V. et al., In:  
DNA Cloning Techniques: A Practical Approach, (D.  
20 Glover, ed.) IRL Press, Oxford, Vol. 1, pp. 49-78  
(1985). Crude lysates of cells expressing plasmid  
encoded proteins were made by harvesting cells from  
overnight cultures and resuspending the cells in 10  
mM Tris pH7.5/10 mM EDTA containing 100 ug  
25 lysozyme/ml. After 10 minutes at room temperature,  
SDS was added to a final concentration of 0.5%. A  
protease inhibitor (Trasylol, Boehringer Mannheim)  
was added to all crude lysates at a final concen-  
tration of 0.3%. The crude protein preparations  
30 were electrophoresed on 10% polyacrylamide-SDS  
Laemmli gels and the separate proteins electrophor-

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etically transferred to nitrocellulose. Laemmli,  
U.K., Nature, 227:680-685 (1970). Towbin, H. et  
al., Proceedings of the National Academy of  
Sciences, USA, 76:4350-4354 (1979). The immobilized  
05 proteins were reacted with a 1:1000 dilution of  
monoclonal antibody IT-13 in TBST overnight at 4°C.  
The nitrocellulose filters were then washed, reacted  
with peroxidase-conjugated goat anti-mouse immuno-  
globulin, and developed as described by Niman and  
10 co-workers. Niman, H.L. et al., Proceedings of the  
National Academy of Sciences, USA, 80:4949-4953  
(1983).

The sequences of 5'-end-labeled restriction  
fragments of the mycobacterial DNA were determined  
15 by a modification of the partial chemical  
degradation technique of Maxam and Gilbert. Brow,  
M.A.D. et al., Mol. Biol. Evol., 2:1-12 (1985).  
Maxam, A.M. and W. Gilbert, Proceedings of the  
National Academy of Sciences, USA, 74:560-564  
20 (1976). For the M13/dideoxy sequencing studies,  
Sau3AI fragments from the mycobacterial DNA inserts  
were subcloned into the BamHI site of M13mp9. Phage  
DNA was isolated from the M13 recombinants and  
subjected to the dideoxy chain termination  
25 sequencing reactions. Biggin, M.D. et al.,  
Proceedings of the National Academy of Sciences,  
USA, 80:3963-3965 (1983). Sanger, F. et al.,  
Journal of Molecular Biology, 143:161-178 (1980).  
The products of the sequencing reactions were  
30 electrophoresed on 6% acrylamide/7M urea/0.5-2.5 x  
TBE gradient sequencing gels. The gels were dried

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under vacuum and exposed to Kodak XRP-1 film. The nucleotide sequences were determined independently for both strands of the mycobacterial DNA.

Computer-aided analyses of the nucleic acid  
05 sequences and deduced protein sequences were performed using the Databases and programs provided by the Nucleic Acid and Protein Identification Resources of the National Institutes of Health as well as the programs of Chou and Fasman and Hopp and  
10 Woods. Chou, P.Y. and G.D. Fasman, Adv. Enzym.,  
47:45-148 (1978). Hopp, T.P. and K.P. Woods,  
Proceedings of the National Academy of Sciences,  
USA, 78:3824-3828 (1981). The nucleotide sequence  
15 of the region containing the M. tuberculosis 65kD gene and the deduced amino acid sequence of the two long open reading frames are represented in Figure 8.

B-galactosidase assays were also carried out. Cells were grown in LB broth or LB broth plus 2.5mM  
20 IPTG to an OD<sub>600</sub> of about 0.3. Crude lysates were made and b-galactosidase activity assayed as described by Miller. Miller, J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1972).

25 Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to the specific materials and components described herein. Such 30 equivalents are intended to be encompassed in the scope of the following claims.

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CLAIMS

1. Isolated DNA encoding an immunogenic protein antigen of Mycobacterium tuberculosis.
2. DNA of Claim 1 selected from the group consisting of DNA encoding Mycobacterium tuberculosis protein antigens of molecular weight 71kD, 65kD, 19kD, 14kD and 12kD.
3. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 65kD and recognized by a monoclonal antibody selected from the group consisting of: IT-31; C1.1; IIH9; IIC8; T2.3; Y1-2; SA2.D7C and IT-13.
4. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 19kD and recognized by a monoclonal antibody selected from the group consisting of: IT-10; IT-12; IT-16; and IT-19.
5. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 71kD and recognized by the monoclonal antibody IT-11.

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6. Isolated DNA encoding an antigenic determinant of Mycobacterium tuberculosis protein.
7. DNA of Claim 6 which encodes an antigenic determinant selected from the group consisting of antigenic determinants of Mycobacterium tuberculosis proteins of molecular weight 71kD, 65kD, 19kD, 14kD and 12kD.
8. Isolated DNA encoding an amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein, said protein having a molecular weight of approximately 65kD.
9. Isolated Mycobacterium tuberculosis DNA encoding an immunodominant protein antigen having a molecular weight of approximately 65kD, said DNA selected from the group consisting of:
  - a. the DNA insert of clone Y3141;
  - b. the DNA insert of clone Y3143;
  - c. the DNA insert of clone Y3150;
  - d. the DNA insert of clone Y3253; and
  - e. the DNA insert of clone Y3262.
10. A protein antigen encoded by DNA of Claim 9.
11. A protein antigen of Claim 10, wherein the protein antigen is recognized by a monoclonal antibody selected from the group consisting of IT-31; C1.1; IIH9; IIC8; T2.3; Y1-2; SA2.D7C and IT-13.

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- 05 12. Isolated DNA having a nucleotide sequence selected from the group consisting of: a) the nucleotide sequence represented in Figure 6, or a portion thereof; b) the nucleotide sequence represented in Figure 7, or a portion thereof; and c) the nucleotide sequence represented in Figure 8, or a portion thereof.
- 10 13. A protein or a peptide selected from the group consisting of: a) proteins or peptides encoded by the nucleotide sequence represented in Figure 6, or a portion thereof; b) proteins or peptides encoded by the nucleotide sequence represented in Figure 7, or a portion thereof; and c) proteins or peptides encoded by the nucleotide sequence represented in Figure 8, or a portion thereof.
- 15 14. A peptide having the amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein, said antigenic determinant being unique to Mycobacterium tuberculosis protein.
- 20 15. A peptide encoded by isolated Mycobacterium tuberculosis DNA, said peptide recognized by helper T cells.
- 25 16. A peptide encoded by the Mycobacterium tuberculosis DNA insert of clone Y3150 or a portion of said DNA insert.

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17. Isolated DNA encoding a protein of Mycobacterium africanum the protein having a molecular weight of 65kD.
18. Isolated DNA encoding a protein of Mycobacterium avium, the protein having a molecular weight of 65kD.
19. A vaccine comprising DNA encoding Mycobacterium tuberculosis protein in a recombinant vaccine vector capable of expressing said DNA.
20. A vaccine of Claim 19 in which the recombinant vaccine vector is vaccinia virus or cultivatable mycobacteria.
21. A vaccine of Claim 20 in which the DNA encodes the 65kD Mycobacterium tuberculosis protein recognized by the monoclonal antibody IT-13, or a portion of said protein.
22. A vaccine comprising DNA encoding an antigenic determinant unique to Mycobacterium tuberculosis cultivatable mycobacteria capable of expressing said DNA.
23. A method of detecting antibody against Mycobacterium tuberculosis in a biological fluid, comprising the steps of:
  - a) incubating an immunoadsorbent comprising a solid phase to which is attached

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- 05 immunodeterminant Mycobacterium tuberculosis protein with a sample of the biological fluid to be tested, under conditions which allow the anti-Mycobacterium tuberculosis antibody in the sample to bind to the immunoadsorbent;
- 10 b) separating the immunoadsorbent from the sample; and
- c) determining if antibody is bound to the immunoadsorbent, as an indication of anti-Mycobacterium tuberculosis in the sample.
24. A method of Claim 23 in which the Mycobacterium tuberculosis protein attached to the solid phase has a molecular weight of approximately 65kD.
- 15 25. A method of detecting antibody against Mycobacterium tuberculosis in a biological fluid, comprising the steps of:
- 20 a) incubating an immunoadsorbent comprising a solid phase to which is attached a peptide having the amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein with a sample of the biological fluid to be tested, under conditions which allow antibody against Mycobacterium tuberculosis to bind to the immunoadsorbent;
- 25 b) separating the immunoadsorbent; and
- c) determining if antibody is bound to the immunoadsorbent, as an indication of the

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presence of the antibody against Mycobacterium tuberculosis in the sample.

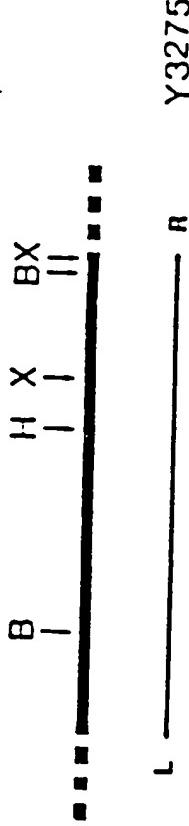
26. A method of Claim 25 in which the peptide has  
the amino acid sequence of an antigenic deter-  
minant which is unique to Mycobacterium  
tuberculosis protein.  
05
27. A kit useful in detecting antibody against  
Mycobacterium tuberculosis in a biological  
fluid, comprising a collection of reagents for  
immunoassay of said antibody, said collection  
10 of reagents a solid phase to which is attached  
immunodeterminant Mycobacterium tuberculosis  
protein or a peptide having the amino acid  
sequence of an antigenic determinant of  
15 Mycobacterium tuberculosis.

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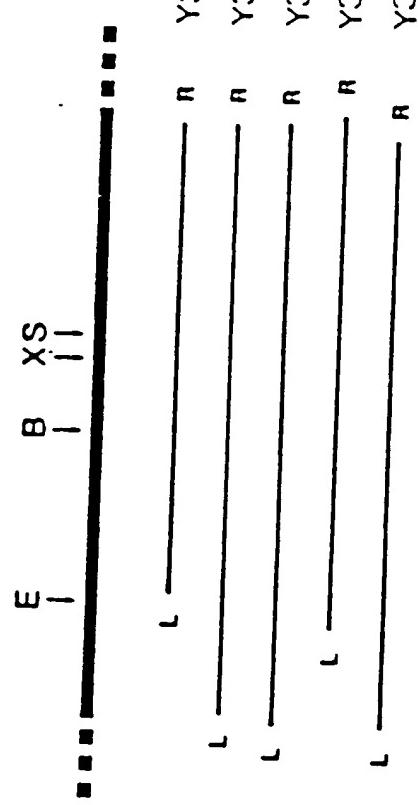
FIGURE 1

 $\mu\text{kb}$ 

12K



14K



19K

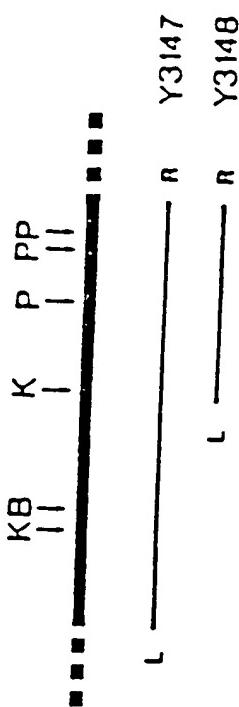


FIGURE 1 (Cont'd)

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$\mu$ Kb<sub>1</sub>

65 K

XBX<sub>III</sub>  
KAA  
E  
A

R ————— L Y3141  
R ————— L Y3143  
L ————— R Y3150  
L ————— R Y3253  
R ————— L Y3262

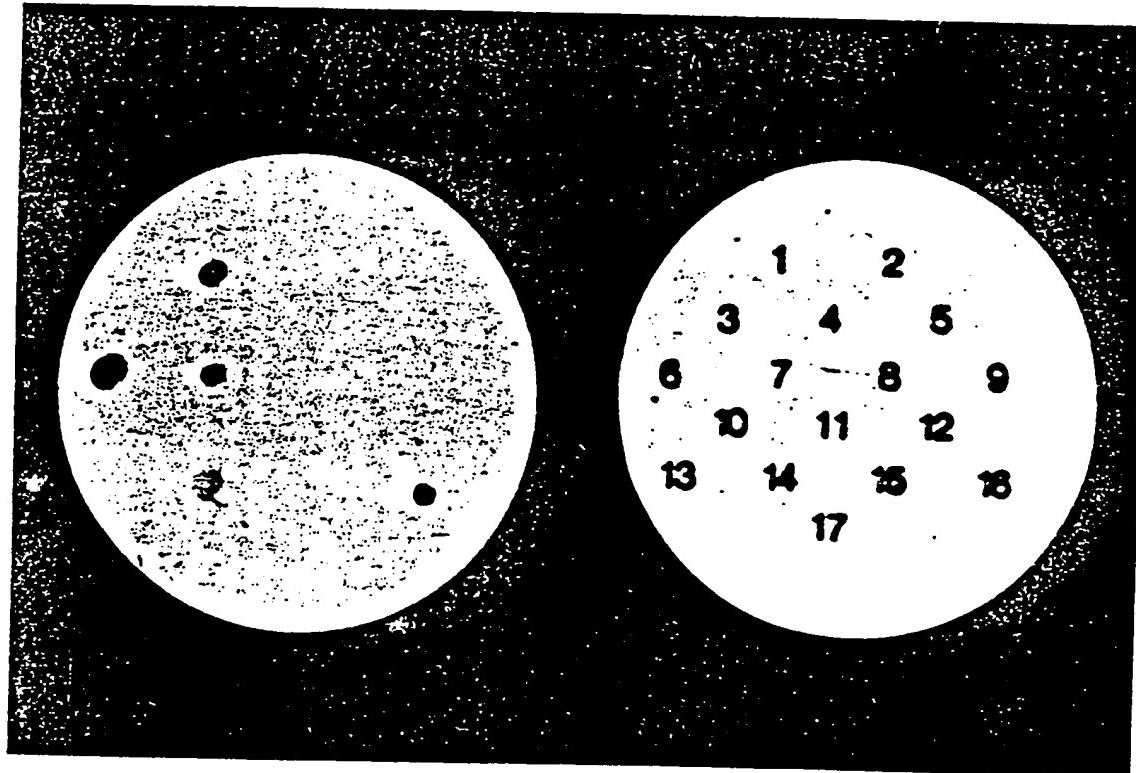
71 K

K X BK G PB E E

R ————— L Y3268  
R ————— L Y3271  
R ————— L Y3272  
R ————— L Y3273

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FIG.2



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FIG.3

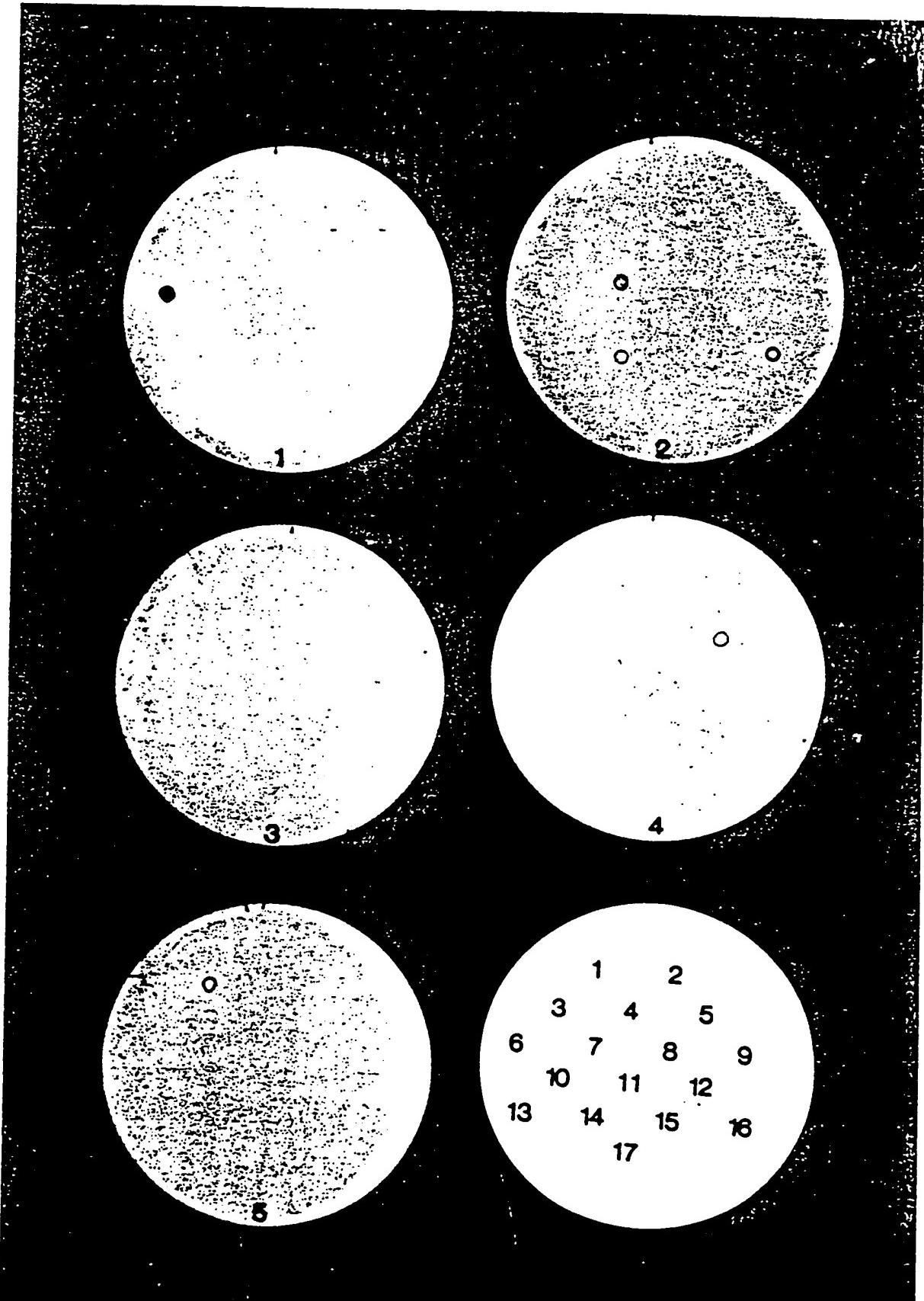
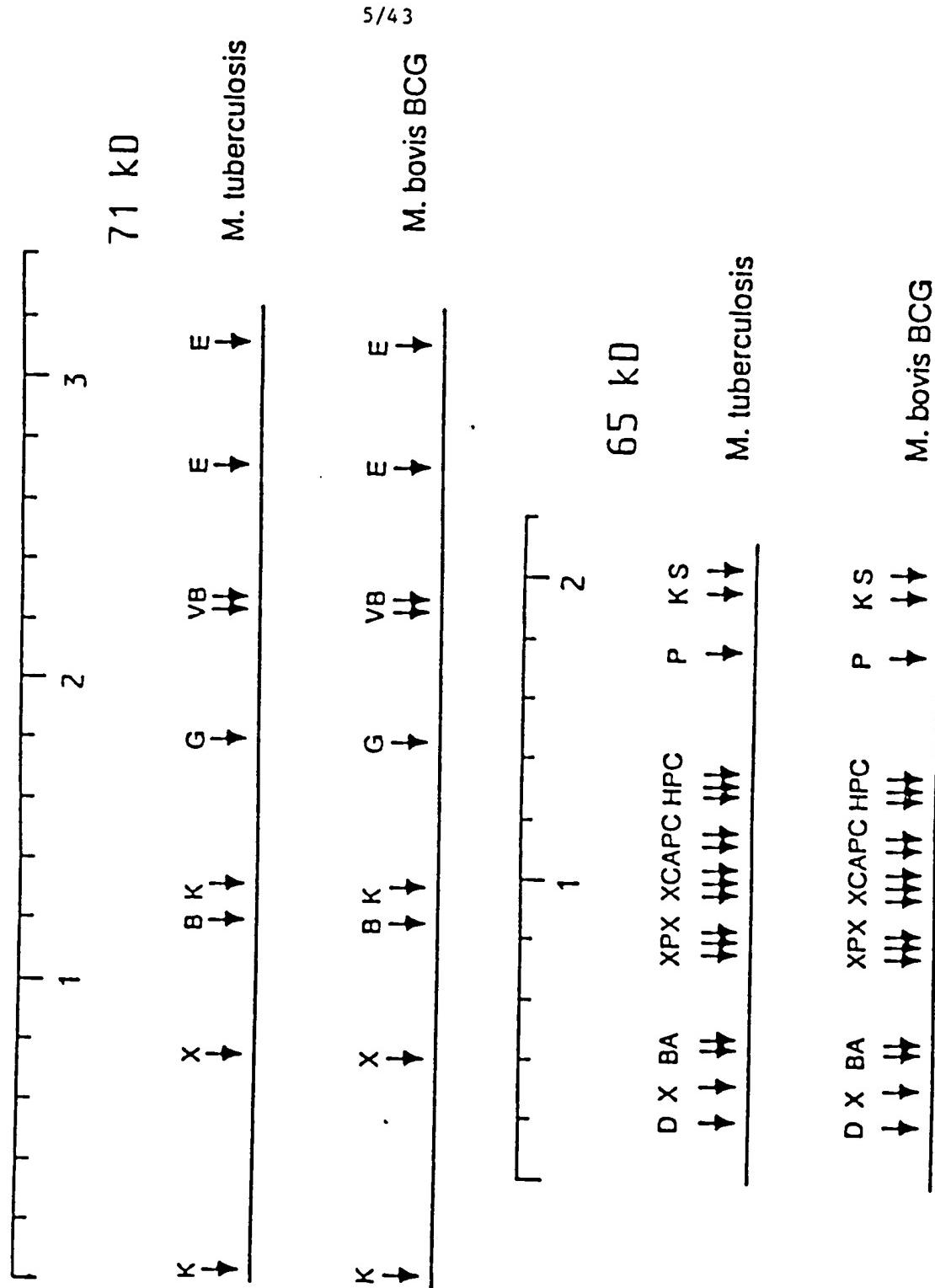


FIGURE 4



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FIGURE 4 (Cont'd)

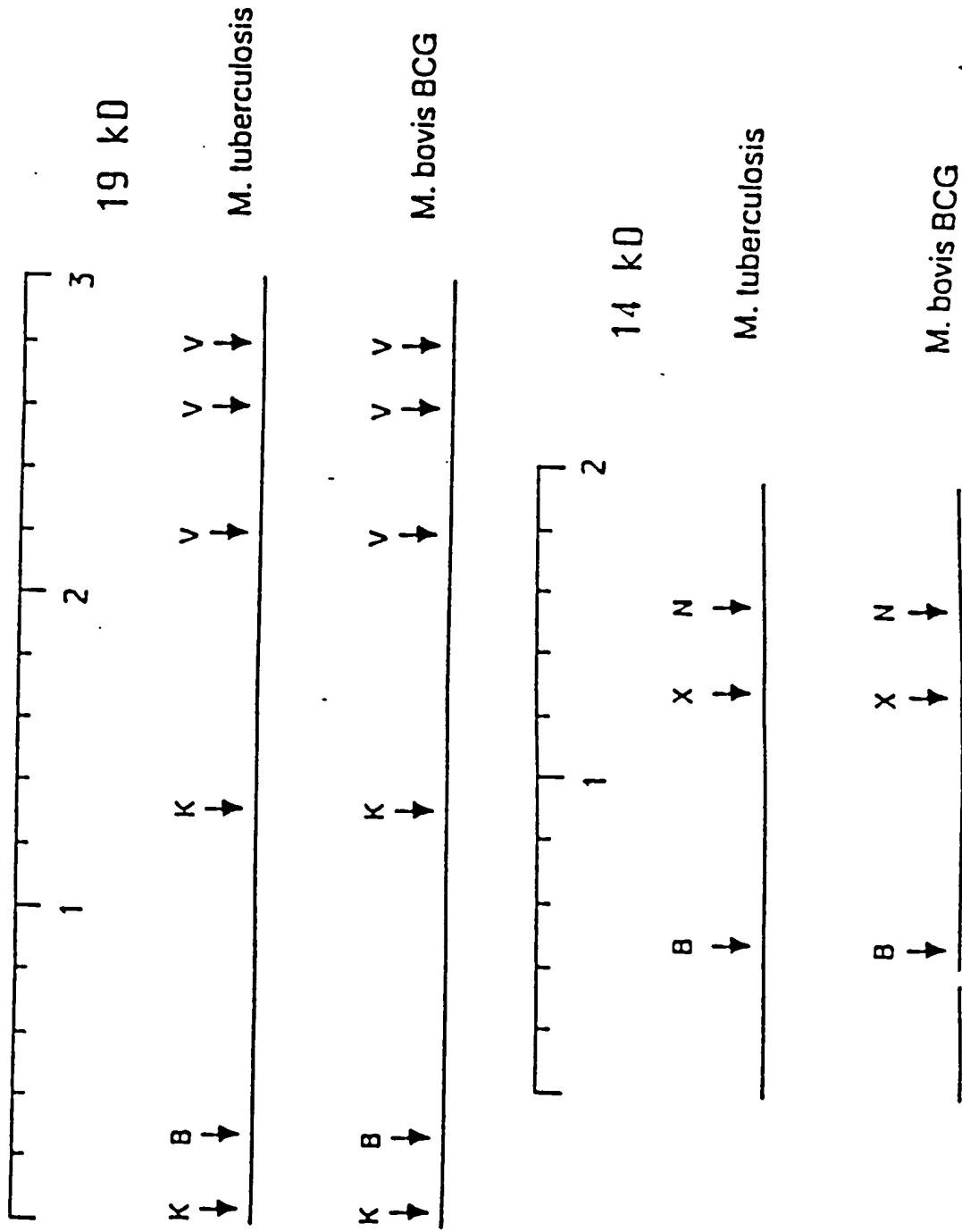


FIGURE 6

F R V N R L G E I A R P G A R T A H Q G  
 S V S I A S V R \* P D Q A R G S R T K A  
 P C Q S P R \* D S P T R R A D R A P R R  
 TTCCGTGTCAATCGGCTCGGTGAGATAAGCCGACCAAGGC  
 10 20 30 40 50 60  
 AAGGCACAGTTAGGGAGGCCACTCTAGGGCTGGTCCGGCCTAGCGCGTGGTCCG  
 E T D I A E T L Y G S W A R P D R V L A  
 G H \* D G R H S L G V L R A S R A G L R  
 R T L R R P S I A R G P A R T A C W P A  
 A A Q G Q Q V V Q V M R G V F G H A Q R  
 P R R V S R L C R L C V A F S A M H N A  
 R A G S A G C A G Y A W R F R P C T T R  
 GCCGGCAGGGTCAGCAGGGTTGTGCAGGTATGGGTGGCCATGCCAACGCG  
 70 80 90 100 110 120  
 CGGGCGGTCCAGTCCAAACACGTCAAATACGCACCGCAAAGCCGGTACGGTGGC  
 G R L T L L N H L N H T A N E A M C L A  
 R A P D A P Q A P \* A H R K R G H V V R  
 A C P \* C T T C T I R P T K P W A C R A

FIGURE 6 (CONT'D)

A E A R E I E V H L R R G L G A R G H L  
 R K R G K \* K C I S A G A S V P G A I W  
 G S A G N R S A S P Q G P R C P G P S G  
 GCGGAAGGGGGAAATAGAAGTGCATCTCCGCAAGGGCCTCGGTGCCGGGCCATCTG  
 130 140 150 160 170 180  
 CGCCTTCGCCCTTATCTCACGTAAGGGGTCCCCGGAGCCACGGGGGGTAGAC  
 R F R P F Y F H M E A P A E T G P A M Q  
 P L A P F L L A D G C P G R H G P G D P  
 S A R S I S T C R R P R P A R P W R S 9/43  
  
 E L D L H P V D G V G L P G L G D V D R  
 N S I S T P S M V W V S P V S V M S T V  
 T R S P P R R W C G S P R S R \* C R P S  
 GAACTCGATCTCCACCCCGTCGATGGTGTGGTCTCGGTGATGTCGACCCT  
 190 200 210 220 230 240  
 CTTGAGCTAGAGGTGGGCAACACCCAGAGGGCCAGAGCTGGCA  
 F E I E V G D I T H T E G T E T I D V T  
 V R D G G R R H H P D G R D R H H R G D  
 S S R W G T S P T P R G P R P S T S R R

FIGURE 6 (CONT'D)

R H D E R N L T G R Q C L P E A A A D V  
 G T T S E T S P V D S V C P R P Q P T C  
 A R R A K P H R S T V S A R G R S R R A  
 CGGCACGAGCGAAACCTCACCGGTGACAGTGTCTGCCGGAGGCCGACGTG 10/43  
 250 260 270 280 290 300  
 GCCGTTGGCTGGCTTGGAGTGGCCAGGTCACAGACGGCTCCGGCTGGCAC  
 P V V L S V E G T S L T Q G L G C G V H  
 A R R A F G \* R D V T D A R P R L R R A  
 C S S R F R V P R C H R G S A A S T G  
 P P E T A R Q H G A V H V A R T A H H R  
 P R R P R A N T V P Y M \* P A R R I I A  
 P G D R A P T R C R T C S P H G A S S P  
 CCCCGGAGACCGGCCAACACGGTGGCGTACATGTAGCCCCGACGGGCATCATCGC  
 310 320 330 340 350 360  
 GGGGGCCTCTGGCGCGGTTGTGCCACGGCATGTACATGGCGTAGTAGCG  
 G R L G R A L V T G Y M Y G A R R M M A  
 G P S R A G V R H R V H L G C P A D D G  
 G S V A R W C P A T C T A R V A C \* R R

FIGURE 6 (CONT'D)

R A G V D V F L H G V R G E P L R R Q H  
 E P A \* M F S C T A C A V N P S G A S T  
 S R R R C F P A R R \* T P P A P A P P  
 CGAGCCGGCGTAGATGTTCCCTGCACGGCGTGGCAGGCAC  
 370 380 390 400 410 420  
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 GCTCGGCCATCTACAAAGGACGTCGGCACGGCCACTTGGGGAGGGCGGGTGTG  
 S G A Y I N E Q V A H A T F G E P A L V  
 L R R L H K G A R R A R H V G G A G A G  
 A P T S T K R C P T R P S G R R R W C R

R H L S R V H V G L G G D A E H P T E M  
 A T F P A S T S A W V V T P S T P P K \*  
 P P F P R P R P G W \* R R A P H R N D  
 CGCCACCTTCCCCGGTCCACGTCGGCCTGGGTGAGCAGCCACCCACCGAAATG  
 430 440 450 460 470 480  
 GCGGTGGAAAGGGCCAGGGCAGCCACCTGCGGGCTCGTGGGGTGGCTTAC  
 A V K G A D V D A Q T T V G L V G G F H  
 G G K G R G R R G P H H R R A G W R F S  
 W R E R T W T P R P P S A S C G V S I I

FIGURE 6 (CONT'D)

I D M A V G V D D R D H G A V G S A V G  
 S T W L W V \* M T A T T G R S A P R W A  
 R H G C G C R \* P R P R G G R L R G G R  
 ATCGACATGGCTGTGGGTGATGACCGCGACCAACGGGGCGTCCGGGTGGC  
 490 500 510 520 530 540 12/43  
 TAGCTGTACCGACACCCACATCTACTGGGCTGGTGGCCAGGCCACCCG  
 D V H S H T Y I V A V V P R D A G R H A  
 R C P Q P H L H G R G R P P R S R P P R  
 S M A T P T S S R S W P A T P E A T P A  
 A I Q V Q R G G G H L G G H Q R V D D D  
 R Y K S S A A A A T S V D T N G S M T I  
 D T S P A R R P R W T P T G R \* R S  
 GCGATAAAGTCCAGCGCGGGGCCAACCTCGGTGGACACCAAACGGGTGGATGACGAT  
 550 560 570 580 590 600  
 CGCTATGTTCAAGGTCTGGCGCCGGGGAGCCACCTGTGGCTACTGCTA  
 R Y L D L A A A V E T S V L P D I V I  
 S V L G A R R R G G R H V G V P R H R D  
 I C T W R P P W R P P C W R T S S S \*

FIGURE 6 (CONT'D)

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|  |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
|--|-----|-----|-----|-----|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|--|
| Q  | P   | S   | V   | T   | L   | N | E | A | D | I | G | D | I | E | S | A | D | L | I | * |  |
| A  | Q   | C   | H   | P   | Q   | R | S | * | Y | W | R | Y | R | I | R | G | P | D | R | * |  |
| CAGCCCCAGTGTACCCCTCAACGAAAGCTGATAATTGGAGATATCGAATCCGGGACCTGATA |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| 610  | 620 | 630 | 640 | 650 | 660 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| GTCGGGGTACAGTGGGAGTTGCTCGACTATAACCTCTATAGCTTAGGCCCTGGACTAT     |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| L  | G   | T   | D   | G   | E   | V | F | S | I | N | S | I | D | F | G | R | V | Q | Y |   |  |
| A  | W   | H   | *   | G   | *   | G | * | R | L | Q | Y | Q | L | Y | R | I | R | P | G | S |  |
| G  | L   | T   | V   | R   | L   | S | A | S | I | P | S | I | S | D | A | S | R | I | S | L |  |
| D  | A   | R   | H   | H   | L   | V | E | A | L | F | R | G | Q | L | G | L | P | P | Q |   |  |
| M  | P   | G   | T   | T   | W   | * | R | P | C | F | A | V | S | W | D | C | R | H | R |   |  |
| C  | P   | A   | P   | P   | G   | R | G | P | V | S | R | S | A | G | I | A | A | T | G |   |  |
| GATGCCGGCACACCACCTGGTAGAGGGCTGTTGGCTCAGCTGGATTGCCGCCACAG       |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| 670  | 680 | 690 | 700 | 710 | 720 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| CTACGGGGCGTGGGACCATCTCCGGACAAAGGCCAGTCGACCCCTAACGGCGGTGTC      |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| I  | G   | P   | V   | V   | Q   | Y | L | G | Q | K | A | T | L | Q | S | Q | R | W | L |   |  |
| H  | G   | A   | G   | G   | P   | L | P | G | T | E | R | D | A | P | I | A | V | A | V | P |  |

FIGURE 6 (CONT'D)

A R C W R T S A R N R P \* S P N G G C A

A G M H R C R R G T V E K R V R V V P  
 L G C T D V G A A P S R N E Y A S L S H  
 W D A P M S A R H R R E T S T R R C P T

GCTGGGATGCCGATGTCGGCGGGCACCGTCAAGAAACGAGTACGGCTCGTGTCCCCA  
 730 740 750 760 770 780

CGACCC'TACGTGGCTACAGCCGGCAGCTCTTGCTCATGCCAGCAACAGGGT  
 S P H V S T P A A G D L F S Y A D N D W  
 Q S A G I D A R C R R S V L V R R Q G V  
 P I C R H R R P V T S F R T R T T G C<sup>14/43</sup>

H H A T I G S L D H T R G Q R G N E S A  
 T T R P S A A L I T H G D S A A M N P R  
 P R D H R Q P \* S H T G T A R Q \* I R D

CACCACGGACCATCGGCAGCCTTGATCACACACGGGACAGCGGGCAAATGAAATCCGGC  
 790 800 810 820 830 840

GTTGGCTGGTAGCCGGTCCGGAACTAGTGTGTGCCCCCTGTGGCGGGTTACTAGGGC  
 V V R G D A A K I V C P S L A A I F G R  
 G R S W R C G Q D C V P V A R C H I R S  
 W A V M P L R S \* V R P C R P L S D A I

FIGURE 6 (CONT'D)

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|  |     |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|--|-----|-----|-----|-----|-----|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I  | G   | V   | V   | E   | I   | R   | C | V | M | Q | R | * | R | V | F | T | V | C | R |   |
| S  | A   | S   | S   | K   | S   | V   | V | S | C | N | G | N | E | C | S | P | C | A | A |   |
| R  | R   | R   | R   | N   | P   | L   | C | H | A | T | V | T | S | V | H | R | V | P | P |   |
| ATCGGGCGTCGAAATCCGTTGTCATGCAACGGTAACGAGTGTACCCGTTCACCGTGTGCCGC |     |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|  | 850 | 860 | 870 | 880 | 890 | 900 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| TAGCCGAGCGCTTAGGCAACAGTACCGTTGCATTGCTCACAAAGTGGCACACGGCG       |     |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| D  | A   | D   | D   | F   | D   | T   | T | D | H | L | P | L | S | H | E | G | H | A | A |   |
| R  | R   | R   | R   | F   | G   | N   | H | * | A | V | T | V | L | T | * | R | T | G | G |   |
| P  | T   | T   | S   | I   | R   | Q   | T | M | C | R | Y | R | T | N | V | T | H | R | R |   |
| L  | D   | D   | G   | S   | G   | R   | F | V | F | H | R | H | Y | I | A | T | T | T | V |   |
| W  | M   | T   | A   | V   | G   | G   | L | C | S | I | G | T | T | L | P | L | L | R | C |   |
| G  | *   | R   | Q   | W   | E   | V   | C | V | P | S | A | L | H | C | H | Y | Y | G | A |   |
| CTGGATGACGGCAGTGGGAGGTTGTTCCATGGGCACIACATGCCACTACTACGGTG       |     |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|  | 910 | 920 | 930 | 940 | 950 | 960 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| GACCTACTGCCGTACCCCTCCAAACACAAGGTAGCCGTGATGTAAACGGGTGATGCCAC    |     |     |     |     |     |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Q  | I   | V   | A   | T   | P   | P   | K | H | E | M | P | V | V | N | G | S | S | R | H |   |
| P  | H   | R   | C   | H   | S   | T   | Q | T | G | D | A | S | C | Q | W | * | * | * | P | A |
| S  | S   | P   | L   | P   | L   | N   | T | N | W | R | C | * | M | A | V | V | V | T | C |   |

FIGURE 6 (CONT'D)

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H A G R C R W R T T L P T R K R E E S A
T P V D A V G E P R Y R P E R E N F P P
R R * M P L A N H A T D Q K E R I F R R
CACGCCGGTAGATGCCGTGGCGAACCAACGCTACCGACCAGAAAGAGAATTTCGGCC
970   980   990   1000  1010  1020
GTGCCGCATCTACGGCAACCGCCTGGTGGCGATGGCTGGTCTCTCTAAAGGGCGG
V G T S A T P S G R * R G S L S F K G G
R R Y I G N A F W A V S W F S L I K R R
A P L H R Q R V V S G V L F L S N E A A
A P R P R A L L T R I L P K R S S M P M
H L D L G P C * R A Y C R S G P Q C R W
T * T S G P A N A H T A E A V L N A D G
GCACCTAGACCTCGGGCCCTGCTAACGGCATACTGCCGAGCGGTCTCAATGCCGATG
1030  1040  1050  1060  1070  1080
CGTGGATCTGGAGCCGGGACGGATGGCGTATGACGGCTCCGCCAGGAGTTACGGCTAC
C R S R P G Q * R A Y Q R L P G * H R H

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FIGURE 6 (CONT'D)

V \* V E P G A L A C V A S A T R L A S P  
 G L G R A R S V R M S G F R D E I G I S

D R Y D R Q R S T G \* S V D \* R S R \* P  
 T A T T G K G A Q G E A W T D G R G S R  
 P L R Q A K E H R → V K R G L T V A V A G  
 GACCGCTACGGCACAGGAAAGGAGGAGGAGGAAAGGGTGAAGCGGTGGACTGACGGTCGGTAGCCG  
 1090 1100 1110 1120 1130 1140  
 CTGGCGATGCTGTCCGTTCTCGTCCCACCTTCCGACCTGACTGCCAGGCCATCGGC  
 V A V V P L P A C P S A H V S P R P L R  
 G S R C A F S C L T F R P S V T A T A P  
 R \* S L C L V P H L T S Q R D R Y G S

E P P F W S Q V F P D V Q A T S R L Q E  
 S R H S G R R S F R M F K Q Q V D Y R K  
 A A I L V A G L S G C S S N K S T T G S  
 GAGCCGCAATTCTGGTCCGAGGTCTTCCGGATGTTCAAGCAACAAGTCACTACAGGAA  
 1150 1160 1170 1180 1190 1200  
 CTGGCGGTAAAGAACCGAGCAGGTCCAGAAGGGCTACAAAGTTCTGATGTCCTT  
 L R W E P R L D K R I N L C C T S \* L F  
 A A M R T A P R E P H E L L D V V P L  
 G G N Q D C T K G S T \* A V L R S C S A

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FIGURE 6 (CONT'D)

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|   |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|------|------|------|------|------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A   | V    | R    | P    | R    | P    | R | Q | A | R | R | Q | A | P | P | P | P | G | R |   |
| R   | *    | D    | H    | D    | R    | G | R | H | D | G | K | P | R | R | R | L | R | A | E |
| G   | E    | T    | T    | T    | A    | A | G | T | T | A | S | P | G | A | A | S | G | P | K |
| GCGGTGAGACCACGACGGCACGGCAGGCACGGCAAGCCCCGGCCGCTCCGGGGCGA    |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1210  | 1220 | 1230 | 1240 | 1250 | 1260 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| CGCCACTCTGGTGTGCTGGGCCGGTCCGGCTGCCGCTGGGGCCGGGGCT           |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| R   | H    | S    | W    | S    | R    | P | L | C | S | P | L | G | R | R | R | A | S |   |   |
| P   | S    | V    | V    | V    | A    | A | P | V | V | A | L | G | P | A | A | E | P | G | F |
| T   | L    | G    | R    | G    | R    | C | A | R | R | C | A | G | A | G | G | P | R | L |   |
| R   | S    | S    | S    | T    | V    | R | T | R | T | S | P | A | P | W | C | A | Q | P | R |
| G   | R    | H    | R    | R    | *    | G | P | E | R | H | R | L | R | G | V | H | N | R | G |
| V   | V    | I    | D    | G    | K    | D | Q | N | V | T | G | S | V | V | C | T | T | A | A |
| AGGTCTGTCATCGACGGTAAGGACCAGAACGTCAACGGCTCCGTGGTGTGACAACCGGG |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1270  | 1280 | 1290 | 1300 | 1310 | 1320 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| TCCAGCAGTAGCTGCCATTCTGGTCTTGCAAGTGGCCGAGGGCACCAACCGTGTGGGCC |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| P   | R    | *    | R    | R    | Y    | P | G | S | R | * | R | S | R | P | T | C | L | R | P |
| T   | T    | M    | S    | P    | L    | S | W | F | T | V | P | E | T | T | H | V | V | A | A |
| D   | D    | D    | V    | T    | L    | V | L | V | D | G | A | G | H | H | A | C | G | R | G |

FIGURE 6 (CONT'D)

P A M S T S R S A G R R P A L P P C S P  
 R Q C Q H R D R R G G D R H C R R A H R  
 G N V N I A I G G A A T G I A A V L T D  
 CCGGAAATGTCAACATCGCGATCGGGGGGGACCGGCATTGCCGCGTGCCTCACCG  
 1330 1340 1350 1360 1370 1380  
 GGCGTTACAGTTAGCGCTAGCCCCGGCTGGCCGTAACGGCGCAGGTGGC  
 R C H \* C R S R R P P S R C Q R R A \* R  
 P L T L M A I P P A V P M A A T S V S  
 A I D V D R D A P R R G A N G G H E G V <sup>19/43</sup>  
  
 T A T L R R \* S P L G S V T S T A S R W  
 R Q P S G G E V R W A R \* R Q R R H A G  
 G N P P E V K S V G L G N V N G V T L G  
 ACGGAAACCCCTCGGAGGTGAAGTCCCGTGGCTGGTAACCGTCAACGGCTGG  
 1390 1400 1410 1420 1430 1440  
 TGCCGTTGGAGGGCCACTTCAGGCAAACCCGAGCCATTGCAGTTGCCGAGCGACC  
 R C G E P P S T R Q A R Y R \* R R \* A P  
 P L G G S T F D T P S P L T L P T V S P  
 A V R R L H L G N P E T V D V A D R Q S

FIGURE 6 (CONT'D)

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|  |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|--|------|------|------|------|------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D  | T    | R    | R    | A    | P    | D | R | V | T | P | R | Q | P | R | T | A | A | T | T |
| I  | H    | V    | G    | H    | R    | T | G | * | R | L | G | N | Q | G | R | Q | P | L | Q |
| Y  | T    | S    | G    | T    | G    | Q | G | N | A | S | A | T | K | D | G | S | H | Y | K |
| GATACACGGTGGCAGCGGACAGGGTAACGGCTCGGCAACCAAGGACGGCAGGCCACTACA |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1450   | 1460 | 1470 | 1480 | 1490 | 1500 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| CTATGTGCAGCCCCGTGGCCTGTCCCCATTGCGGGAGCCGTTGCTGCCGGTCGGTGATGT |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| I  | C    | T    | P    | C    | R    | V | P | Y | R | R | P | L | W | P | R | C | G | S | C |
| Y  | V    | D    | P    | V    | P    | C | P | L | A | E | A | V | L | S | P | L | W | * | L |
| V  | R    | R    | A    | G    | S    | L | T | V | G | R | C | G | L | V | A | A | V | V | L |
| R  | S    | L    | G    | P    | L    | P | G | S | T | W | P | T | R | C | H | R | * | T | S |
| D  | H    | W    | D    | R    | Y    | R | G | R | H | G | Q | P | D | V | T | G | E | Q | V |
| I  | T    | G    | T    | A    | T    | G | V | D | M | A | N | P | M | S | P | V | N | K | S |
| AGATCACTGGGACCGCTAACGGGTCGACATGGCCAACCCGATGTCAACGGGTGAACAAGT |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1510   | 1520 | 1530 | 1540 | 1550 | 1560 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| TCTAGTGACCCCTGGCGATGGCCCCAGCTGTACAGTGGCCTACAGTGGCCACTGTGTTCA |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| S  | *    | Q    | S    | R    | *    | R | P | R | C | P | W | G | S | T | V | P | S | C | T |
| I  | V    | P    | V    | A    | V    | P | T | S | M | A | L | G | I | D | G | T | F | L | D |
| D  | S    | P    | G    | S    | G    | P | D | V | H | G | V | R | H | * | R | H | V | L | R |

FIGURE 6 (CONT'D)

R S K S R \* P V P N L K R V D A G C E Q  
 V R N R G D L F I T \* S M R A V N S  
 F E I E V T C S ] \* P K A C R C G L \* T A  
 CGTTGAAATCGAGGTGACCTAACCTAAAGCGTGTGATGCCGGCTGTGAAACAG  
 1570 1590 1600 1610 1620  
 GCAAGCTTAGCTCCACTGGACAAGGATTGGATTTCGCACAGCTAACGCCGACACTTGTC  
 T R F R P S R N R V \* L T D I R A T F L  
 N S I S T V Q E \* G L A H R H P S H V A 21/43  
 E F D L H G T G L R F R T S A P Q S C R 3  
 R V G A G Q S G L A R R F E R L P S V  
 A S E P G S Q A \* R G D D S S G C H P S  
 R R S R A V R P S A A T I R A V A I R Q  
 CGCGTCGGAGCCGGCAGTCAGGCCTAGCGGGCACGATTCGAGCGGTGCCCCATCCGTC  
 1630 1640 1650 1660 1670 1680  
 GCGCAGCCTCGGCCGTCAAGTCCGGATCGGCCAAGCTCGCTAACGGTAGGCAG  
 A D S G P L \* A \* R P S S E L P Q W G D  
 R R L R A T L G L A A V I R A T A M R \*  
 T P A P C D P R A R R N S R N G D T L

FIGURE 6 (CONT'D)

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|   |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|------|------|------|------|------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| K   | W    | Q    | P    | H    | R    | K | L | G | I | S | G | * | A | T | H | G | D | R | S |
| S   | G    | N    | R    | T    | A    | N | S | V | Y | P | G | E | L | L | T | V | I | V | P |
| V   | A    | T    | A    | P    | Q    | T | R | Y | I | R | V | S | Y | S | R | * | S | F | R |
| AAGTGGCAACCGCACCGCAAACTCGGTATATCCGGTACTCAGGTGATCGTTCC     |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1690  | 1700 | 1710 | 1720 | 1730 | 1740 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| TTCACCGTTGGCGTTTGAGCCATATAAGGCCACTCGATGAGTGGCCACTAGCAAAGG |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| L   | P    | L    | R    | V    | A    | F | E | T | Y | G | P | S | S | V | T | I | T | G |   |
| T   | A    | V    | A    | G    | C    | V | R | Y | I | R | T | L | * | E | R | H | D | N | R |
| H   | C    | G    | C    | R    | L    | S | P | I | D | P | H | A | V | * | P | S | R | E | T |
| V   | V    | R    | L    | D    | H    | S | G | D | D | R | Q | A | E | P | G | A | T | G | L |
| L   | C    | A    | L    | T    | T    | A | E | T | I | A | R | P | S | P | V | L | P | A | W |
| C   | A    | P    | *    | P    | Q    | R | R | S | P | G | R | A | R | C | Y | R | L | G |   |
| GTTGTGGCCTTGACACAGCGGAGACGATGCCAGGGCGAGCCCCGGCTAACGGCTTG  |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1750  | 1760 | 1770 | 1780 | 1790 | 1800 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| CAACACGGAAACTGGTGTGGCCTCTGCTAGCGGGCTGGGCCACGATGGCCGGAAC   |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| N   | H    | A    | K    | V    | A    | S | V | I | A | L | G | L | G | T | S | G | A | Q |   |
| Q   | A    | G    | Q    | G    | C    | R | L | R | D | G | P | R | A | R | * | R | S | P |   |
| T   | R    | R    | S    | W    | L    | P | S | S | R | W | A | S | G | P | A | V | P | K | A |

FIGURE 6 (CONT'D)

A G P \* R I A A G E P L E N L G L Q R G  
 R D R D V S P R A N R S K T S D C S A A  
 G T V T Y R R G R T A R K P R T A A R P  
 CGGGACCGTGTGACGGTATGCCGGGGCAACCGCTCGAAAACCTCGGACTTCAGCGGGC  
 1810 1820 1830 1840 1850 1860  
 CGCCCTGGCACTGCATAGCGGCCGGCGCTGGAGCTTTGGAGCCTGACCGTGGCGCCG  
 R S R S T D G R A F R E F V E S Q L A A  
 P V T V Y R R P R V A R F G R V A A R G  
 P G H R I A A P S G S S F R P S C R P R G<sup>23/43</sup>  
  
 R N T R P I V D H L Q H D V R R P G A Q  
 G I P G P L S I T C S T T C V G P V L K  
 E Y P A H C R S P A A R R A S A R C S S  
 CGGAATACCCGCCATTGTCGATCACCTGCAGCACGACGTGCCTGGCCGGTGGCTCAA  
 1870 1880 1890 1900 1910 1920  
 GCCTTATGGGGGTAACAGCTAGTGGACGTGCTGCACGCCAGCGAGTT  
 P I G P G N D I V Q L V V H T P G T S L  
 S Y G A W Q R D G A A R R A D A R H E L  
 F V R G M T S \* R C C S T R R G P A \* A

FIGURE 6 (CONT'D)

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|   |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|------|------|------|------|------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A   | R    | R    | H    | D    | R    | A | G | T | G | V | H | A | G | V | G | Q | Q | V | G |
| R   | V    | V    | T    | I    | V    | P | G | P | V | C | T | R | A | L | A | S | R | L | V |
| A   | S    | S    | R    | S    | C    | R | D | R | C | A | R | G | R | W | P | A | G | W | S |
| GCGCGTCACTGGATCGTGCCGGACCGGTGTGCACGCCGGTGGCCAGCAGGTTGGT     |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1930  | 1940 | 1950 | 1960 | 1970 | 1980 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| CGCGCAGCAGTAGCACCGGCCACACGTCGCCACACGGTGTGCCAACCGGTGTCCAACCA |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| R   | T    | T    | V    | I    | T    | G | P | G | T | H | V | R | A | N | A | L | L | N | T |
| A   | D    | D    | R    | D    | H    | R | S | R | H | A | R | P | R | Q | G | A | P | Q | D |
| R   | R    | *    | S    | R    | A    | P | V | P | T | C | A | P | T | P | W | C | T | P | * |
|   |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| H   | H    | L    | V    | Q    | P    | C | R | I | T | R | D | D | H | R | F | G | G | Q | V |
| T   | T    | W    | C    | N    | R    | A | A | S | P | G | M | T | T | G | S | G | G | R | S |
| P   | P    | G    | A    | T    | V    | P | H | H | P | G | * | P | P | V | R | G | A | G | R |
| CACACCTGGTGCACCCGTGCCATCACCCGGATGACCACCCGGTGTGGGGCAGGTC     |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1990  | 2000 | 2010 | 2020 | 2030 | 2040 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| GTGGGACCACTGGCACGGCGTAGTGGGCCCTACTGGTGGCCAAAGCCCCGGTCCAG    |      |      |      |      |      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| V   | V    | Q    | H    | L    | R    | A | A | D | G | P | I | V | V | P | E | P | P | L | D |
| G   | G    | P    | A    | V    | T    | G | C | * | G | P | H | G | G | T | R | P | A | P | R |
| W   | R    | T    | C    | G    | H    | R | M | V | R | S | S | W | R | N | P | P | C | T | S |

FIGURE 6 (CONT'D)

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E R P L V I W S G N M S V A D R V N R K
S A H W * S G P A T * A S L T A S T A S
A P T G D L V R Q H E R R * P R Q P Q A
GAGGGCCCACTGGTGAATCTGGTCCGGAAACATGAGCGCTGACCGGTCAACCGCAAG
2050 2060 2070 2080 2090 2100
CTCCGGGTGACCACTAGACCAGGCCGTTGACTCGCAGCGACTGGCCAGTGGCGTTTC
L A W Q H D P G A V H A D S V A D V A L
A G V P S R T R C C S R R Q G R * G C A
R G S T I Q D P L M L T A S R T L R L G
P R H V H R S A F Q R P P R V Q T R Q Q
R D M S T G P R S S G R P E S R R A S S
A T C P P V R V P A A A P S P D A P A A
CCGGACATGTCCACCGGTTCCAGGGCCCCGAGTCCAGACGCCAGCAG
2110 2120 2130 2140 2150 2160
GGCGCTGTACAGGGCCAGGCCAAGGTGGCCGGGGCTCAGGTCTGCGGGTCTGCGTC
R S M D V P G R E L P R G S D L R A L L
A V H G G T R T G A A A G L G S A G A A
R C T W R D A N W R G G R T W V R W C C

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FIGURE 6 (CONT'D)

Q Q V L D E T C Y P L G L R C H P A H R  
 S R S S T R R V I R S V S D A T R L I A  
 A G P R R D V L S A R S P M P P G S S R  
 CAGCAGGTCCCTCGACGAGACGTGTTATCCGCTCGGTCTCCGATGCCACCCGGCTCATCGC  
 2170 2180 2190 2200 2210 2220  
 GTCTGGAGGCTGCTCTGCACAATTAGGCCAGAGGCCAGGGCTACGGTGGGCCAGTAGCG  
 L L D E V L R T I R E T E S A V R S M A  
 A P G R R S T N D A R D G I G G P E D R  
 C T R S S V H \* G S P R R H W G A \* R T<sup>26/43</sup>  
 V C D G L G I V P Y P L R Q F R V T T D  
 C A T A S G S S P I R C V N S V \* P R I  
 V R R P R D R P L S A A S I P C N H G S  
 GTGTGCGACGGCCTCGGGATCGTCCCTATCCGCTGGCTCAATTCCGTTAACCCACGGAT  
 2230 2240 2250 2260 2270 2280  
 CACACGCTGCCGGAGCCCTAGCAGGGATAGGGCACATTGGTGCCTA  
 H A V A E P D D G I R Q T L E T Y G R I  
 T R R G R S R G R D A A D I G H L W P D  
 H S P R P I T G \* G S R \* N R T V V S R

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FIGURE 6 (CONT'D)

R P E L P H L G G V C V R F G H P D R  
 D P S C P T S V E G F A S G S G T R T G  
 T R V A P P R W R G L R Q V R A P G P V  
 CGACCCGAGTTGCCAACCTCGGTGGGGTTCAGGTTGGCACCCGGACCCGG  
 2410 2420 2430 2440 2450 2460  
 GCTGGCTCAACGGGGTGGAGCCACCTCCCCAAACGCAGTCCAAGCCCAGTGGCCTGGCC  
 S G L Q G V E T S P N A D P E P V R V P  
 V R T A G G R H L P K R \* T R A G P G T  
 G S N G W R P P T Q T L N P C G S R Y<sup>28/43</sup>  
 \* L D L A A I Q R \* V D D F A R G L R D  
 S L T S P R S N G R S T T S L A V C A T  
 A \* P R R D P T V G R R L R S R F A R P  
 TAGCTTGACCTGCCGGATCCAACGGTAGGTGACACTTCGCTCGGGTTGGCGGAC  
 2470 2480 2490 2500 2510 2520  
 ATCGAACTGGAGGGGGCTAGGGTAGCTGCCATCCAGCTGGCTGAAGGGAAACGGCGCTG  
 L K V E G R D L P L D V V E S A T Q A V  
 A Q G R R S G V T P R R S R E R N A R G  
 S S R A A I W R Y T S S K A R P K R S R

FIGURE 6 (CONT'D)

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|   |      |      |      |      |      |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
|---|------|------|------|------|------|---|---|---|---|---|---|---|---|----|---|---|---|---|---|
| R   | R    | N    | G    | A    | S    | A | R | L | M | M | T | I | P | 'A | V | V | A | A | T |
| A   | A    | T    | A    | P    | A    | P | A | * | * | * | R | F | R | R  | S | S | R | R | P |
| P   | Q    | R    | R    | Q    | R    | P | L | D | D | D | S | G | G | R  | R | G | D | Q |   |
| CGCCGCAACGGGCCAGCGCCCCGCTTGATGACGATTCCGGCGTGCAGCGAC                 |      |      |      |      |      |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
| 2530  | 2540 | 2550 | 2560 | 2570 | 2580 |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
| GCGGGCTTGCCGGTCGGCGAACACTACTGCTTAAGGCCAGCAGGCCGCTGG                 |      |      |      |      |      |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
| A   | A    | V    | A    | G    | A    | Q | H | H | R | N | R | R | D | D  | R | R | G |   |   |
| G   | C    | R    | R    | W    | R    | G | S | S | S | S | E | P | P | R  | R | P | S | W |   |
| R   | L    | P    | A    | L    | A    | R | K | I | I | V | I | G | A | T  | T | A | A | V | L |
|   |      |      |      |      |      |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
| N   | A    | I    | T    | V    | T    | I | P | K | M | I | S | I | C | N  | I | V | A | S | T |
| T   | Q    | S    | P    | *    | R    | F | R | K | * | S | A | S | A | T  | S | W | R | R | R |
| R   | N    | H    | R    | D    | D    | S | E | N | D | Q | H | L | Q | H  | R | G | V | D | V |
| AACGCAATCACCGTAGCGATTCCGAAATGATCAGCAATCTGCAACATCGTGGCGTCGACG        |      |      |      |      |      |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
| 2590  | 2600 | 2610 | 2620 | 2630 | 2640 |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
| TTGCGTTAGTGGCACTGCTTAAGGCTTTACTAGTCGGTAGACGTTAGCAGCGCTGC            |      |      |      |      |      |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
| V   | C    | D    | G    | H    | R    | N | R | F | H | D | A | D | A | V  | D | H | R | R | R |
| R   | L    | *    | R    | S    | S    | E | S | F | S | * | C | R | C | C  | R | P | T | S | T |
| A   | I    | V    | T    | V    | I    | G | F | I | I | L | M | Q | L | M  | T | A | D | V | N |
|   |      |      |      |      |      |   |   |   |   |   |   |   |   |    |   |   |   |   |   |
| L   | P    | I    | D    | R    | P    | V | T | M | T | S | C | P | F | R  | L | G | A | A | S |
| C   | P    | S    | T    | G    | R    | * | R | * | R | R | A | R | F | G  | S | E | R | P | A |
| A   | H    | R    | Q    | A    | G    | D | D | V | V | P | V | S | A | R  | S | G | Q | H |   |
| TTGGCCCATCGACAGGCCGGITGACGGATGACGTCTGGCTCGGCCGTTTCGGGCTCGGAGGGCCAGC |      |      |      |      |      |   |   |   |   |   |   |   |   |    |   |   |   |   |   |

FIGURE 7

1 GAA TTC CAA CCG TCG GTG CAG ATC CAG GTC TAT CAG GGG GAG CGT GAG 48  
Glu Phe Gln Pro Ser Val Gln Ile Gln Val Tyr Gln Gly Glu Arg Glu

49 ATC GCC GCG CAC AAC AAG TTG CTC GGG TCC TTC GAG CTG ACC GCC ATC 96  
Ile Ala Ala His Asn Lys Leu Leu Gly Ser Phe Glu Leu Thr Gly Ile

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97 CCG CCG CGG CCG GGG ATT CCG CAG ATC GAG GTC ACT TTC GAC ATC 144  
Pro Pro Ala Pro Arg Gly Ile Pro Gln Ile Glu Val Thr Phe Asp Ile

145 GAC GCC AAC GGC ATT GTG CAC GTC ACC GCC AAG GAC AAG GCC ACC GGC 192  
Asp Ala Asn Gly Ile Val His Val Thr Ala Lys Asp Lys Gly Thr Gly

FIGURE 7 (CONT'D)

193 AAG GAG AAC ACG ATC CGA ATC CAG GAA GGC TCG GGC CTG TCC AAG GAA 240  
Lys Glu Asn Thr Ile Arg Ile Gln Glu Gly Ser Gly Leu Ser Lys Glu

241 GAC ATT GAC CGC ATG ATC AAG GAC GCC GAA GCG CAC GCC GAG GAG GAT 288  
Asp Ile Asp Arg MET Ile Lys Asp Ala Glu Ala His Ala Glu Glu Asp 31/43

289 CGC AAG CGT CGC GAG GAG GCC GAT GTT CGT AAT CAA GCC GAG ACA TTG 336  
Arg Lys Arg Arg Glu Glu Ala Asp Val Arg Asn Gln Ala Glu Thr Leu

337 GTC TAC CAG ACC GAG AAG TTC GTC AAA GAA CAG CGT GAG GCC GAG GGT 384  
Val Tyr Gln Thr Glu Lys Phe Val Lys Glu Gln Arg Glu Ala Glu Gly

FIGURE 7 (CONT'D)

385    GGT TCG AAG GTA CCT GAA GAC ACG CTG AAC AAG GTT GAT GCC GCG GTG    432  
Gly Ser Lys Val Pro Glu Asp Thr Leu Asn Lys Val Asp Ala Ala Val

433    GCG GAA GCG GAA GGC GGC ACT TGG CCG ATC GGA TAT TTC GGC CAT CAA    480  
Ala Glu Ala Glu Gly Gly Thr Trp Arg Ile Gly Tyr Phe Gly His Gln

481    GTC GGC GAT GCA GAA GCT GGG CCA GGA GTC GCA GGC TCT GGG GCA AGC    528  
Val Gly Asp Gly Glu Ala Gly Pro Gly Val Ala Gly Ser Gly Ala Ser

529    GAT CTA CGA AGC AGC TCA GGC TGC GTC ACA GGC CAC TGG CGC TGC CCA    576  
Asp Leu Arg Ser Ser Ser Gly Cys Val Thr Gly His Trp Arg Cys Pro

577    CCC CGG CGG CGA GCC GGG CGG TGC CCA CCC CGG CTC GGC    615  
Pro Arg Arg Arg Ala Gly Arg Cys Pro Pro Arg Leu Gly

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## FIGURE 8

5' TCGAACGGGGGGTGAACCCGGTCTTGCACTCGGCATAGGCCATAGGGGAGTGCTAAG  
 3, AGCTTGTCCCCGACTGGCCCACCTGGCTTGGCTACCTGGCAACGTAACGGTATCCGCTACGGATT  
 10 20 30 40 50 60

AATAACCGTTGGCACTCGGGACCCGGTAGGTGAGTCGGCTAGGTGGGACGGTGAGGCCAGGGCCAG  
 TTATTGCAAACCGTGGCTGGCCACTCAGGATCCAGCCACTTGGCAACTTGGCAAGGGCAGGGGGGG  
 70 80 90 100 110 120

GTCGGAGGGAGTGGCAGGGACAACATTGAGCCGGTCCGTCGGGGCACTGGCCGGGG  
 CAGCGTGGCTACCGTGGCTGGCTGGCTGGCAACTGGCAAGGGGGGGGG  
 130 140 150 160 170 180

\* R G C R H P V T P V S S P I R R  
 AGCGTAAGTAGCCGGGTTGGCGTACCCGGTGAACCCGGTTCACTCCCGATC  
 TCGCATTCACTGGCCAAACGGCAGTGGCCACTGGGGAAAGTAGGGGCTAGGGCT  
 190 200 210 220 230 240

N H F A M A K T I A Y D E E A R R G L E  
 ATCACTTCGCAATGGCCAAGACAATTGGTACCGACGAAGAGGGGGCTCGAGC  
 TAGTGAAGCGTTACCGGTCTGGTAAACGGCATGGTTCGCTTC  
 250 260 270 280 290 300

R G L N A L A D A V K V T L G P K G R N  
 GGGCTTAACGGCCCTGGGATGGGTAAGGGTACATTGGGCCCCAAGGGGGCAAGC  
 CCCCGAACITGGGAGGGCTACGGCAATTCCACTGTAAACCGGGGT  
 310 320 330 340 350 360

V V L E K K W G A P T I T N D G V S I A  
 TCGTCCTGGAAAAGAAGTGGGGTGCCCCACCGATACCAACGATGGTGTGTC  
 AGCAGGACCTTCTTCAACGGGGCTAGTGGGGCTAGTGGCTACACAGGTAGGGT  
 370 380 390 400 410 420

FIGURE 8 (CONT'D)

FIGURE 8 (CONT'D)

E S N T F G L Q L E L T E G M R F D K G  
 AGTCCAAACACTTTGGCTCGAGCTCACCGAGGCTATGGGTTCGACAAGGGCT  
 TCAGGTTGGAAACCCGACCTCGAGCTCCAGTGGCTCCCATAACGCCAAGCTGTCCGA  
 790 800 810 820 830 840  
  
 Y I S G Y F V T D P E R Q E A V L E D P  
 ACATCTCGGGTACTTCGTGACCGACCCGGAGCGTCCAGGAGGGCTCCTGGAGACCCCT  
 TGTAGAGCCCCATGAAGCACTGGCTGGGCTCGGACTGGCACTGGCTGGGGA  
 850 860 870 880 890 900  
  
 Y I L L V S S K V S T V K D L L P L L E  
 ACATCCTGGCTAGCTCCAGGTCAAGGTGCTCCACTGTCAAGGATCTGCTGGCTGGAGA  
 TGTAGGGACGACCACTGGAGCTCGAGGTTCCACAGGTGACAGTCCCTAGACGGACGGAGCTCT  
 910 920 930 940 950 960  
  
 K V I G A K P L L I I A E D V E G E A  
 AGGTCACTGGAGGGTAAGGCCGCTGCTGATCATCGCCAGGACCGTGGAGGGCAGGGCC  
 TCCAGTAGCCCTCGGCCATTGGGCAACGACTAGTAGCCGCTCCGGCTCCGGG  
 970 980 990 1000 1010 1020  
  
 L S T L V V N K I R G T F K S V A V K A  
 TGTCCACCCCTGGTCCGTCAACAAAGATCCGGCACCTTCAGTGGGTCAAGGGCTC  
 ACAGGCTGGACCAAGCACTGGTCTAGGGGGTGGAACTTCAGCCACGGGAGTCCGAG  
 1030 1040 1050 1060 1070 1080  
  
 P G F G D R R K A M L Q D M A I L T G G  
 CCGGCTTGGGACCCGCAAGGGGATGGTGCAGGATATGGCCATTCTCACGGGTGGTC  
 GGGCGAAGCCGGTGGGGCTTCCGGTACGACGTCCTAACCGGTAAGAGTGCCCCACCA  
 1090 1100 1110 1120 1130 1140  
  
 Q V I S E E V G L T L E N A D L S L L G  
 AGGTGATCAGCGAAGGGTGGGCTTGACGGGACCTGTGGCTGGCTAGGCA  
 TCCACTAGTCGCTTCCAGGGGACTGGCACCTTGGGCTGGACAGGGACGGATCCGT  
 1150 1160 1170 1180 1190 1200

FIGURE 8 (CONT'D)

K A R K V V T K D E T T I V E G A G D  
 AGGCCCGCAAGGTCTGGTCACCAAGGACCGACCATCCTCGAAGGGGGGGTGACA  
 TCCGGGGTTCCAGCACCGTGGTCTGGCTCTGGTGGTAGCAGCTCCGGGCCACTGT  
 1210 1220 1230 1240 1250 1260  
  
 T D A I A G R V A Q I R Q E I E N S D S  
 CCGACGCCATGCCGGACCGAGTGGCCAGATCCGCCAGGAGATCGAGAACAGCGACTCCG  
 GGCTGCCGTAGCGGGCTTGCTCACGGGTCTAGGGGTCTAGCTCTAGCTCTAGCTGGCTGAGGC  
 1270 1280 1290 1300 1310 1320  
  
 D Y D R E K L Q E R L A K L A G G V A V  
 ACTACQACCCGTGAGAAAGCTGCCAGGGCTGGCCAGCTGGCTGGGTGTCGGCGGTGA  
 TGATGCTGGCACTCTTGACGTCCCTGCCGACCCGGTTGACCCGGCCACACAGGGCACT  
 1330 1340 1350 1360 1370 1380  
  
 I K A G A A T E V E L K E R K H R I E D  
 TCAAGGGGGTCCCCCACCGAGGTGCAACTCAAGGAGGGCAAGCAGGAGGATG  
 AGTTCCGGCAAGGGGTGGCTCCAGCTTGAGTTCCAGCTGGCTGGCTAGCTCTAC  
 1390 1400 1410 1420 1430 1440  
  
 A V R N A K A A V E E G I V A G G G V T  
 CGGTTCGCAAATGCCAAGGGCCGGCGTGGAGGGCATCGTGGGGGTGTCGACGC  
 GCCAAGCGTTACGGTTCCGGGGCGAGCTCCCGTAGCAGGGCCACCCACACTGGC  
 1450 1460 1470 1480 1490 1500  
  
 L L Q A A P T L D E L K L E G D E A T G  
 TGTGCAAGCGGG  
 ACAACGTTGG  
 1610 1620 1630 1640 1650 1660  
  
 A N I V K V A L E A P L K Q I A F N S G  
 CCAACATCGTGAAGGTGGGGCTGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGG  
 GGTGTAAGCACCTCCACCCGGACCTCCGGGGGGGGGGGGGGGGGGGGGGGGGG  
 1570 1580 1590 1600 1610 1620

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FIGURE 8 (CONT'D)

L E P G V V A E K V R N L P A G H G L N  
TGGAGCCGGCGTGGTGGCCGAGAAGGTGCCAACCTGGCCTGGCCACGGACTGAACG  
ACCTGGGGCACCCGGCTTCCACGGCGTTGGACGGGTGGCTGACTTGC  
1680 1660 1850 1840

A Q T G V Y E D L L A A G V A D P V K V  
CTCAGACCCGGTGTCTACGGGATCTGCTCGCTGGCTGACCCGGTCAAGGGTGA  
GAGTCTGGCCACAGATGCTCCCTAGACGAGCGACGGCAACGACTGGCCAGTCCACT  
1740 1690 1700 1710

T R S A L Q N A A S I A G L F L T T E A  
CCC GTTGGCGCTGCAGAAATGCCGGCTCCATGCCGGCTGTTCCCTGACCACCGAGGCC  
GGCAAGGCCGGCACGTTACGCCAGGTAGGCCGGCACAGGACTGGTGCTCCGGC  
1800 1750 1760 1770

V V A D K P E K E K A S V P G G D M G  
TCGTTGCCGACAAGCCGGAAAAGGAGAAGGGCTTCCGGTCCGGCATGGCTG  
AGCAAACGGCTGTTGGCTTTCCCTCTTCCAGGGCAAGGGCAGGGCTGTACCCAC  
1850 1810 1820 1830

G M D F \*  
GCATGGATTCTGACCCCCGGAGAAGTCCCAGGAGGAGCCGGTCCCTTGTGGGGCC  
CGTACCTAAAGACTGGGGGGCTCTCAGGGCTGGCTTCAAGGGCAAGGGAAACACCCGG  
1880 1870 1890 1900

GGGCTCCTGGGAGCTACGGTACCGAGAACACCAACGGCAGTGTAGGCAACCTT  
CCCAGGAGACCAACCCCTCGATGCCATGGCTCTTGTGGCTCAGCACATCCGGTGGAA  
1980 1930 1940 1950

TGGCCGCTGTGGGGAGTGGCGCTCTCGGTGCAGCAGCCGGATGGGTACGA  
ACGGGACACCCGGCTCAGCCCCGGCAAGGCCACGGTCCGTGGCTACCCATGCT  
2040 2030 2020 2010

1990 1990 1990 1990

FIGURE 8 (CONT'D)

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FIGURE 8 (CONT'D)

2530      2540      2550      2560      2570      2580  
 CGCAAGCCCCGAGTCGTGGCTGCCCGAGTTGACCGAACCTCGGGTAGCTGGCTGCCAGGGCT  
 GCGGTTCCGGCTCAGCAACGGACGGGCTAACTGCTTCCGAGGCCATGGACCACGGTCCGA  
 R L G S D N S G H V F S P Y S T G P S  
  
 2590      2600      2610      2620      2630      2640  
 TCTAAGCCCCGGGTTGGCCGGGGAGCCAGGCCGGGACTGGCCGCTACCGGGGTTGGCT  
 AGATTCCGGGCCCCAAACGGGGCTCGGTGGGGATGGGGCAAGGCCAA  
 R L G P N A G S G A A S G S G P H P N  
  
 2650      2660      2670      2680      2690      2700  
 CCTGACTCCAGGGCCCAACAGGAGGCACTGGCCGGGGGACTGGGGCTGTTGGTCAG  
 CGGACTCAGGTCCGGGGGGTTGTCCTCGGTGACCGGGGGGCTGGGGCACAAACAGTC  
 G S D L G G V P A S A P A A V P T N T L  
  
 2710      2720      2730      2740      2750      2760      2780  
 CCCCGAGTTGAGGACGTTGGCCAGGGCGTGTGGAGACCCGGGCTTGATCCGAGGGGGCA  
 CGGGCTCAACTCCTGCAAGGGCTCCGGACAACCTCTGGGGGGCAACTAGGGCTCCGGCT  
 G S N L V N A L G H Q L G G T S G L A S  
  
 2770      2780      2790      2800      2810      2820  
 CGCGAGGAATGCCCGAACCTCAAAGCCCGCTCATGCCGGCTGGCGTAGCCGGGGGA  
 CGGCTCCCTACGGGCTTGAGTTGGCTTGGGGGACCGAGTACGGGGCACGGGCT  
 A L I G S S L A A T S M G G T A Y G A S  
  
 2830      2840      2850      2860      2870      2880  
 GCTGACCAAGGGCCGCCTCCGAGGCCAGCCCCGGCTTCTTAAGGGGGCTTTTGCATCCCC  
 CGACTGGTTCGGCTGGGGAGGGATTCGGGGGGAAAGGAAACTAACAGGGCT  
 S V L A E S G A A S G L A H Q M G A  
  
 2890      2900      2910      2920      2930      2940  
 GTTCCAGAAGCTGGGTGTTGAGGGCTGGCTGCCAGGGGGCTGGATTGTCCCCGA  
 CAAGGTCTTGGACCAACTCCGACGGGACGGGACTGGCAACTAACAGGGCT  
 H W F S T H L S G A S G L G A N I T G S

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FIGURE 8 (CONT'D)

2960      2980      2970      2980      2990      3000  
 GGTCCCGATGCCGCTGTTCAAGGGAGCCCCGAATTCCCGATGCCGATGCTTCCGCTGCCGGA  
 CCAGGGCTACGGGACAACTCCCTGGCTTAAGGGCTACGGCTACAAAGGGACGGCCT  
 T   G   I   N   L   S   G   S   H   G   T   G   I   N   G   S   G   S  
 3010      3020      3030      3040      3050      3060  
 GTTGAATAAGCCGACCGTGGCGGGAGTTCCCGAAGCCGATGTTGCCGCTACCCGA  
 CAACTTATCGGCTGCACGGCCACGGCTCAAGGGCTTGGCTACAAACGGCGATGGCCT  
 N   F   L   G   V   N   G   T   G   S   N   G   F   G   I   N   G   S   G   S  
 3070      3080      3090      3100      3110      3120  
 GTTGAAGCCGGAAACCCATCTGGTGAATCACCGGCTGATCCCGAACCCGATATTCCCGCT  
 CAACTTCGGGGCTTGGTAGACCACCTAGTGGCCACTAGGGCTTGGCTATAAGGGCGA  
 N   F   G   G   F   G   M   Q   H   D   G   T   I   G   F   G   I   N   G   S  
 3130      3140      3150      3160      3170      3180  
 ACCGGTGTGGCGAACGGCATATTCCCGTCCGGAGGGTGGCCAGGGCTCCAAACGGCGA  
 TGGCCACAACGGCTTGGCTATAAGGGCAGGGCTCCAAACGGCGAACGGCGA  
 G   T   N   G   F   G   I   N   G   D   G   L   N   G   L   N   G   S  
 3190      3200      3210      3220      3230      3240  
 GCCGGTGTGGCGCTGCCGATGTTGGCTGCCGGTGTGGCTGCCGATGTTGTTGTTGTT  
 CGGCCACAACGGGACGGCTACAAACGGCACGGCCACGGCCACAAACAAACAAACAA  
 G   T   N   G   S   G   I   N   G   T   G   I   N   G   S   G   I   N   H   N  
 3250      3260      3270      3280      3290      3300  
 CGCGATGTTGTTGGCGATGTTGTTGCCGATGTTGCCGCTGCCGGTGTGGCGAA  
 CGCTACAAACAACGGCTACAAACAACGGCTACAAACGGCTACAAACGGCGAACGGCTT  
 G   I   N   N   G   I   N   N   G   I   N   G   S   G   T   N   G   F  
 3310      3320      3330      3340      3350      3360  
 GCCCAGATTGATCTGGGGTTTGGCGATGTCGATGCCGAGGGTCCGCAAGGACCTGCTG  
 CGGGTCTAACTAGACGGGCAAGAACGGCTACAGCTACGGCTACGGCTTCTGGACGGAC  
 G   L   N   I   Q   G   N   K   G   I   D   I   G   L   N   R   L   V   Q   .Q

FIGURE 8 (CONT'D)

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|  |  |      |      |      |
|--|--|------|------|------|
| 3370   | 3380   | 3390 | 3400 | 3410 |
| CCAGGGCCCCAGTTGCGCACGGCATCGAAGTGGTAACCAAGCCATCGGCCATCGCCGC       | GGTCCCCGGTCAACACGGCTGGGGTCTGGGTAGCTTCACCATGGTGGTAGGGCG     |      |      | 3420 |
| W P A L Q A V A A S D F H Y G A M A A                            |  |      |      |      |
| 3430   | 3440   | 3450 | 3460 | 3470 |
| CACGTCACATGCCAACATTGCTCGTATGCCGCTCGAACGTCATGAGCGCCGGAGCGGT       | GTGAGGTACGGGTGTAACGAGCATAACGGGAGCTGCAGGTACTCGGGCTCGCAA     |      |      | 3480 |
| V D L A W M Q E Y A A E V D M L A P A N                          |  |      |      |      |
| 3490   | 3500   | 3510 | 3520 | 3530 |
| CTGGCCAAACCAGTTCGTAGCTGCCAGCACGCTGCATCAGGCCACCGATTGGCCGTACAC     | GACGGGTTTGGTCAAGCATCGACGGTCTGAGCTAGTCCGGTGGCTAACCGGGATGGTG |      |      | 3540 |
| Q G F W N T A A L L Q M L G R N A A V V                          |  |      |      |      |
| 3560   | 3560   | 3570 | 3580 | 3590 |
| TGCCGGCTGCACGGTGGCCGCCAGCCGGCTCGAACGGGGCTGGCTGTGCTGATGGCCATGGCTG | ACGGGGACGTGCCACGGGGTCCGGGGAGCTGGGGAGAACGGTACGGGAC          |      |      | 3600 |
| A P Q V T A A L A E F A T A M A Q                                |  |      |      |      |
| 3810   | 3820   | 3630 | 3840 | 3850 |
| TGCCGGCCCTTGTCTCCGCCCTGGCTGCCGCCAGGGCTGAGGCCAGGCTAGGTACTGGGTTCG  | ACGGGGCAACAAGGGGAGGGGACGGGACGGGACGGTCCGATCCATGACCCAAACG    |      |      | 3860 |
| A A Q E A Q A A T S L W A L Y Q T A                              |  |      |      |      |
| 3870   | 3880   | 3890 | 3700 | 3710 |
| GACGGCCATCATGCCGGGGAGGGACGGGAGCCAGGGCAACTAGTCAGTTGGATGT          | CTGGGGTAGGGGGCCCTGGCTGGGTGGCTCAAGGCTACAV                   |      |      | 3720 |
| V A M M A A S P G L W A G S T L E S T                            |  |      |      |      |
| 3730   | 3740   | 3750 | 3760 | 3770 |
| GACGGAGCCAAGGGAGGGTATTGACGGCAATTCTTGGCCAGGCTCGGCCAGGGCGT         | CTGGCTCGGTTGGCTGGATAACTGGCTGGTAAAGAAGGGGGTCCGGCA           |      |      | 3780 |
| V S G L S A I S A L L E E A L E G W A T                          |  |      |      |      |

FIGURE 8 (CONT'D)

|      |   |      |      |      |      |      |
|------|---|------|------|------|------|------|
| 3790 | GGCCGCAGCAATTAGGGTCCCCGACCCGGACCGAATTCAAGTGGCGAACCATCAGTGGCGAACATTGATCTC<br>CCGGCGTCGTTAATGCCAGGGCTGGGCCCCTGGGCTGGGAAACTTGTAGTCACGGCTTAACTAGAG<br>A A I L P G S G P G A F M L A S N I E | 3810 | 3800 | 3820 | 3830 | 3840 |
| 3850 | TGGCGGCAACCACGGAAAATGCCGGCTTGTCAGCGGATCCAACCTTAACCTGAGTCAGGGACCC<br>ACGGCGCTTGGCTGGGTTAACGGCGAACAGTCGGCTAGGGTGAATTGACAGTCGCTGGC<br>P P L W A F H P S T L R D L K V T L S R              | 3860 | 3870 | 3880 | 3890 | 3900 |
| 3910 | TTGGCGTGGCGGTATCGGCACCTCAATTACCACTCATCTTGGGTATCTTTGGACCC<br>AACGGCACCGCCATAGGGCGTAGGGCTGAAGTTATGGTGAGTAGAAACCCAGTAGAAACCTCCGG<br>Q R P P V E I G S H K P T M K P A G                    | 3920 | 3930 | 3940 | 3950 | 3960 |
| 3970 | CCTAGGGAACCCGCCAGCTTACCTAGTCCCCGGTAGGGGGGACTGGGGGGGATGGCAGC<br>GGATCCCTGGGGTGGGAATGGATCAAGGGCCATCCCCGGCTGACCCGGCCCTACGTCG<br>R P V A L K G L G P Y P G V P R S A A                      | 3980 | 3990 | 4000 | 4010 | 4020 |
| 4030 | TGAGGGGCTGCCACCTGGCCCGTAATGTCGGCTGGTATGGCAAGGACCGACGGGGGGATGGCAGC<br>ACTCCCCAGACGGCTGGACGGGCATTACAGGGACCATACCCGTTCGTTGGCTGGGGGGGG<br>S P R G G A G Y H R Q Y P L C R R P G              | 4040 | 4050 | 4060 | 4070 | 4080 |
| 4090 | AAGAGTTGCTCCGGGACGGCGTTCAACCCGGTTGATCGAACATGTCGACCGAACCTCACCGACCG<br>TTCTCAACGAGGGCTGGCAAGTGGCCAACTAGCTTGTACAGCTGAGTGGCTG<br>L T A G R R T *  | 4100 | 4110 | 4120 | 4130 | 4140 |
| 4160 | GCCTCACCGACCAACTCGCCCTGCTACCGGCCAACCCCCGACGGGGCTGGCTGGGACCGATGGGAGTGGCTGGTTG<br>CGGAGTGGCTGGGTTGGCTGGGACCGATGGGACCGAACGGCAACAGGATTGGCTGGGTTGGCTGG<br>4170                               | 4180 | 4190 | 4200 |      |      |

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## FIGURE 8 (CONT'D)

|   |      |      |      |      |      |
|---|------|------|------|------|------|
| 4210  | 4220 | 4230 | 4240 | 4250 | 4260 |
| TGCTCTGGCACAGCCCCGGGTGGCAGGATAACAGGTCCGGCCATGTGGCGGGGTGGAAAG  |      |      |      |      | 3'   |
| ACGAGACCGTGTGGGGCCACGTCCCTATATGTCCAGGGGTACACCGGGCACCTTC       |      |      |      |      | 5'   |
|   |      |      |      |      |      |
| 4270  | 4280 | 4290 | 4300 | 4310 | 4320 |
| AGGTGTGGACCCCCGGGACGGTTGGCTGGACCCGGCTTGGGTAGATCTGCCGCCACGGACA |      |      |      |      |      |
| TCCACACCTGGGCTGCCAACCCACCTGGGAAACCCATCTAGACGGGGGGGTGCTGT      |      |      |      |      |      |
|   |      |      |      |      |      |
| 4330  | 4340 | 4350 | 4360 | 4370 |      |
| CCGGATATGGACACCCGTCGGCATGGTGGCGAAGGTACGGGCACCCGGCAGCGGAATTCC  |      |      |      |      |      |
| GGCCCTATACCTGTGGCAGGGCTCCCTACACCGCTTCCATGGGGGTGGCTGCTTAAG     |      |      |      |      |      |

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|---|--|--|--|
| (51) International Patent Classification <sup>4</sup> :<br><br>C12N 15/00, A61K 39/04<br>G01N 33/569  |  | A3   | (11) International Publication Number: WO 88/05823<br><br>(43) International Publication Date: 11 August 1988 (11.08.88) |
| (21) International Application Number: PCT/US88/00281<br><br>(22) International Filing Date: 1 February 1988 (01.02.88)   |  | (74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).  |  |
| (31) Priority Application Number: 010,007<br><br>(32) Priority Date: 2 February 1987 (02.02.87)<br><br>(33) Priority Country: US  |  | (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  |  |
| (71) Applicant: WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US).<br><br>(72) Inventors: HUSSON, Robert, N. : 60 Parkman Street, Brookline, MA 02146 (US). YOUNG, Richard, A. : 11 Sussex Road, Winchester, MA 01890 (US). SHINNICK, Thomas, M. : 1434 Rainier Falls Drive, Atlanta, GA 30329 (US). |  | <p><b>Published</b></p> <p><i>With international search report<br/>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report:<br/>3 November 1988 (03.11.88)</p> |  |

(54) Title: MYCOBACTERIUM TUBERCULOSIS GENES ENCODING PROTEIN ANTIGENS

(57) Abstract

*Mycobacterium tuberculosis* genes encoding five immunologically relevant proteins have been isolated by systematically screening a lambda gt11 recombinant DNA expression library with a collection of murine monoclonal antibodies directed against protein antigens of this pathogen. One of the *M. tuberculosis* antigens, a 65kD protein, has been shown to have determinants common to *M. tuberculosis* and *M. leprae*. In addition, genes encoding proteins of other mycobacteria (*M. africanum*, *M. smegmatis*, *M. bovis* BCG and *M. avium*) have been isolated. Isolation and characterization of genes encoding major protein antigens of *M. tuberculosis* make it possible to develop reagents useful in the diagnosis, prevention and treatment of tuberculosis. They can be used, for example, in the development of skin tests, serodiagnostic tests and vaccines specific for tuberculosis.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 88/00281

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>8</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: C 12 N 15/00; A 61 K 39/04; G 01 N 33/569

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

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## III. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category <sup>9</sup> | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>  | Relevant to Claim No. <sup>13</sup> |
|-----------------------|---|-------------------------------------|
| X                     | Proc. Natl. Acad. Sci. USA, volume 82, May 1985,<br>R.A. Young et al.: "Dissection of Mycobacterium tuberculosis antigens using recombinant DNA", pages 2583-2587<br>see the whole document cited in the application  | 1,2,4,6,<br>7,12-15,<br>19,23,25-27 |
| Y                     | --  | 3,8-11,20-22,24                     |
| X                     | Infection and Immunity, volume 51, no. 2, February 1986, American Society for Microbiology, (Washington, DC, US), H.D. Engers et al.: "Results of a World Health Organization-sponsored workshop to characterize antigens recognized by Mycobacterium-specific monoclonal antibodies", pages 718-720<br>see page 718, column 2, line 8 - page 719, column 1; tables I,II cited in the application | 1-16,19,<br>23-27                   |
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## IV. CERTIFICATION

Date of the Actual Completion of the International Search

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| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |  |                        |
|--|--|------------------------|
| Category   | Citation of Document, with indication, where appropriate, of the relevant passages   | Relevant to Claim No   |
| X  | Chemical Abstracts, volume 105, no. 19,<br>10 November 1986, (Columbus, Ohio, US),<br>S. Bhattacharya et al.: "Expression of<br>Mycobacterium tuberculosis genes in<br>Escherichia coli", see page 198,<br>abstract 166229t, & J. Biosci. 1986,<br>10(2), 277-81<br>--   | 1-3,6-16,<br>23-27     |
| X  | Biological Abstracts/RRM, no. 30116549,<br>T.M. Buchanan et al.: "Recombinant<br>Escherichia-colis for expression and<br>production of species-specific<br>epitopes of Mycobacterium-leprae and<br>Mycobacterium-tuberculosis", see<br>title and terms,<br>& Abstracts of the Annual Meeting of<br>the American Society for Microbiology<br>(US), 1986, vol. 86, no. 0, p. 122<br>-- | 14                     |
| X  | Biological Abstracts, volume 82, no. 2,<br>1986, (Philadelphia, PA, US),<br>B.R. Bloom et al.: "Genes for the<br>protein antigens of the tuberculosis<br>and leprosy bacilli", see page AB-532,<br>abstract 14488, & Biosci. Rep. 5<br>(10/11): 839-846, 1985<br>--  | 1-27                   |
| Y  | Nature, volume 316, 1 August 1985,<br>(London, GB),<br>R.A. Young et al.: "Genes for the<br>major protein antigens of the<br>leprosy parasite Mycobacterium<br>leprae", pages 450-452<br>see the whole document<br>cited in the application<br>--  | 3,8-11,16,<br>19-22,24 |
| Y  | Nature, volume 319, no. 6048, 2 January<br>1986, (London, GB),<br>A.S. Mustafa et al.: "Human T-cell<br>clones recognize a major M. leprae<br>protein antigen expressed in E. coli",<br>pages 63-66<br>see the whole document<br>cited in the application<br>--  | 15                     |
| A  | Infection and Immunity, volume 49, no. 2,<br>August 1985, American Society for<br>Microbiology,<br>T.P. Gillis et al.: "Immunochemical<br>characterization of a protein<br>associated with Mycobacterium<br>leprae cell wall", pages 371-377<br>--   |                        |
|  |  | . . . . .              |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |  |                      | TY (PCT)  |
|--|--|----------------------|---|
| Category   | Citation of Document, with indication, where appropriate, of the relevant passages   | Relevant to Claim No |   |
| A  | Infection and Immunity, volume 50, no. 3, December 1985, American Society for Microbiology,<br>J.E.R. Thole et al.: "Cloning of Mycobacterium bovis BCG DNA and expression of antigens in Escherichia coli", pages 800-806 cited in the application<br>--  | 1-27                 | D 88/05824<br>988 (11.08.88)<br><br>rtment, BMP and Company,                |
| P,X  | Infection and Immunity, volume 55, no. 6, June 1987, American Society for Microbiology,<br>D.B. Young et al.: "Screening of a recombinant Mycobacterial DNA library with polyclonal antiserum and molecular weight analysis of expressed antigens", pages 1421-1425 see the whole document<br>-- | 1-27                 | t), BE (Euro-<br>DE (European European pa-<br>European pa-<br>pean patent). |
| P,X  | Proc. Natl. Acad. Sci. USA, volume 84, March 1987,<br>R.N. Husson et al.: "Genes for the major protein antigens of Mycobacterium tuberculosis: The etiologic agents of tuberculosis and leprosy share an immunodominant antigen", pages 1679-1683<br>-----                                       | 1-27                 | I glycoprotein<br>doma cell cul-<br>glycoprotein.                           |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.  OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE :

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

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1-16,19-27

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